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The objective of these study was to characterize the anticonvulsant actions mediated by activation of the adenosine receptor in the rat prepiriform cortex (PPC), a forebrain area which may be essential to the seizure generalization. All compounds evaluated were microinjected into the PPC.

The adenosine agonist N-ethylcarboxamidoadenosine (NECA) inhibited kainic acid (KA)-induced seizures in a dose-dependent and highly potent manner; and the anticonvulsant effect of NECA was completely abolished by the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (8-*p*SPT), suggesting that adenosine receptor activation underlies the efficacy of NECA in protecting against KA-induced seizures. The ability of the adenosine agonist NECA and the nucleoside transport blocker dilazep to inhibit the convulsant effects of KA, an agonist for one subtype of glutamate receptors, supports an interaction between adenosine and excitatory amino acid systems.

CGS21680, an A_2-selective adenosine agonist, was the least potent anticonvulsant against bicuculline methiodide (BMI)-induced seizures of the

adenosine analogs tested. Pharmacological characterization revealed a significant correlation between the anticonvulsant potency of adenosine analogs and their affinities for A_1 adenosine receptors. Therefore, the seizure suppressant action of adenosine and adenosine analogs appears to be mediated through the A_1 subtype of adenosine receptors in the PPC.

Manipulation of endogenous adenosine in the PPC was a strategy to affect seizure expression. The adenosine kinase inhibitors and a nucleoside transport blocker were demonstrated to be highly efficacious and potent as anticonvulsants against BMI seizures. In contrast, an adenosine deaminase inhibitors was both less potent and less efficacious. These findings suggest that accumulation of endogenous adenosine may contribute to seizure suppression, and that adenosine kinase and adenosine transport system may play a pivotal role in the regulation of extracellular levels of adenosine. The proconvulsant effects of the adenosine receptor antagonist, 8-pSPT, was observed in both BMI- and KA-seizure models. Moreover, reduction of extracellular adenosine formation by a focal injection of an ecto-5´-nucleotidase inhibitor resulted in convulsions. These results confirm that adenosine is an endogenous antiepileptogenic substance.

Finally, bilateral injection of NECA in the PPC protected against seizures initiated by intravenous infusion of bicuculline, further indicating that adenosine A_1 receptor population in the prepiriform cortex represents a fundamentally important element in modulation of seizure susceptibility in the CNS.

Activation of Adenosine Receptors in Prepiriform Cortex Modulates Seizure Susceptibility

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CHAPTER I

INTRODUCTION

The epilepsies are a family of clinical syndromes that have in common a transient, recurrent, self-sustained interruption of normal brain function and simultaneous hypersynchronous activation of a large population of neurons in one focal area or generally throughout brain (Dichter and Ayala, 1987). Epilepsy is one of the most common afflictions of man. With a prevalence of approximately 1%, it is estimated that 50 million persons worldwide suffer from these neurological disorders (Rogawski and Porter, 1990). Although many are well controlled with available therapies, perhaps one-quarter of the total continue to have seizures. The drugs used to treat epilepsy today are not all that different from the anticonvulsant drugs used in 1950s. Since the introduction of valproate in 1978, no new antiepileptic drug has been approved in the United States for the primary therapy of epilepsy (Rogawski and Porter, 1990). For many of these people the development of new anticonvulsant agents offers the only hope of achieving adequate control of their seizures. To those individuals who are currently being adequately controlled by anticonvulsant medications, drugs with fewer toxic side effects over currently available agents would represent a significant therapeutic advance.

Although much is known about the physiological basis of the abnormal discharges accompanying seizure phenomena, the cellular mechanisms and neuroanatomical pathways responsible for epileptogenesis remain conjectural. The transition to a seizure appears to be due to simultaneous increments in excitatory influences and decrements in inhibitory processes (Dichter and Ayala, 1987). It has been suggested that there may be a primary defect in the neuronal membrane that results in an instability of the resting membrane potential; possible underlying mechanisms include an abnormality of potassium conductance, or a defect in the voltage-sensitive calcium channels, or a deficiency in the membrane ATPases linked to ion transport (Meldrum, 1990). There may be primary defects in the GABAergic inhibitory system or in the sensitivity or arrangement of the receptors involved in excitatory neurotransmission. A positron emission tomography study in man has shown a decrease in benzodiazepine receptor number in the presumed epileptic focus in patients with partial epilepsy (Savic *et al.*, 1988). Recent studies suggest that there may be increases in the receptor density of glutamate and related excitatory transmitters both in children with various types of generalized seizures and in adults with temporal lobe seizures (Gedds *et al.*, 1990; Represa *et al.*, 1989). Electrophysiological studies also provide evidence for hypersensitivity of N-methyl-D-aspartate (NMDA) receptors in the cortex of patients with focal epilepsy (Avoli and Oliver, 1987). Therefore, the different kinds of epilepsy probably arise from different physiological and morphological abnormalities

(Meldrum, 1990) and hence may show differential responses to anticonvulsant medications (Rogawski and Porter, 1990).

The endogenous purine substance, adenosine, has been shown to be a major inhibitory neuromodulator, and to play important roles in regulation of excitatory transmission in many aspects to maintain neuron homeostasis (Synder, 1985). Loss of the endogenous anticonvulsant mechanisms may contribute to certain seizure events and amplification of adenosine system may be a useful approach in the treatment of epilepsy (Dragunow, 1988 and 1991).

In order to better understand the role of adenosine involvement in modulation of neuronal activity in the CNS, this introductory chapter will briefly review: (1) adenosine formation and metabolism in tissues, (2) adenosine receptor subtypes, (3) the neuromodulatory actions of adenosine in the central nervous system, and (4) the piriform cortex, a brain region which may be associated with both epileptogenesis and the anticonvulsant action of adenosine.

Adenosine Formation and Metabolism

The schematic illustration of metabolic pathways and transport for adenosine, a participant in both intracellular and extracellular pools, is shown in Fig.I-1. Intracellular adenosine formation is mainly through two pathways either from 5´-AMP via the action of cytoplasmic 5´-nucleotidase or from S-adenosylhomocysteine (SAH) via the action of SAH hydrolase. Extracellular adenosine can be released from an intracellular source or produced by membrane-bound 5´-nucleotidase (ecto-5´-nucleotidase) which converts 5´-AMP

to adenosine. Adenosine degradation is mainly dependent on the activities of two enzymes, adenosine kinase and adenosine deaminase. The adenosine release and uptake appears to occur at least in part on a bidirectional, facilitated-diffusional transporter for nucleosides (Paterson *et al.*, 1985; White and McDonald, 1990). In addition to the facilitated-diffusion process, a high affinity, Na^+-dependent, active transport system for adenosine has recently been described in rat brain cells (Johnston and Geiger, 1989).

Under normal physiological conditions, very little intracellular adenosine is formed via the cytoplasmic 5´-nucleotidase pathway because the enzyme is strongly inhibited by normal cytoplasmic levels of ATP and ADP (Schrader, 1983). Alternatively, adenosine production comes from the transmethylation reactions via SAH hydrolase. Intracellular adenosine formed through this pathway is rapidly shuttled back to ATP by a series phosphorylation reactions initiated by adenosine kinase to keep low levels of adenosine in the metabolic pools. In the neural tissue, the part of extracellular adenosine may be attributed to the breakdown of ATP which can be co-released with neurotransmitters (White and McDonald, 1990).

During periods of hypoxia or increased workload, the cellular energy charge changes such that intracellular levels of ATP and ADP are decreased. Thus the removal of the inhibitory factors over cytoplasmic 5´-nucleotidase results in a dramatic production of adenosine (Schrader, 1983). It has been well documented that the extracellular adenosine level is markedly elevated, for

example in brain, in response to various pathophysiogical stimuli such as ischemia, hypoxia, trauma, seizures and exposure to excitatory amino acids (Van Wylen *et al.*, 1986; Hagberg *et al.*, 1987; Phillis *et al.*, 1988; Ballarin *et al.,* 1991; Winn *et al.*, 1980; Jhamandas and Dumbrille, 1980). Although adenosine in traumatized tissue may serve a homeostatic protective function, the capacity of this system to raise adenosine levels is limited due to the short half-life of extracellular adenosine (Marangos and Miller, 1991). Endogenously released adenosine is rapidly taken up by neural and glial cells and followed by intracellular metabolism via phosphorylation and deamination (Deckert *et al.*, 1988). Therapeutic strategies may involve more prolonged stimulation of the adenosine system in brain via an increase in its half-life; this might therefore be viewed as a potentiation or amplification of beneficial homeostatic mechanisms that will be useful in the treatment of seizures, ischemia, stroke and generalized head trauma (Marangos and Miller, 1991; Rudolphi, 1991).

Adenosine Receptors

The effects of adenosine appear to be mediated by specific membrane-bound receptor proteins. Adenosine receptors belong to the purine receptor family, which is divided into P_1 (adenosine) and P_2 (ATP or ADP) receptors (Burnstock, 1978). P_1 receptors preferentially bind adenosine over adenine nucleotides and are competitively blocked methylxanthines such as caffeine and theophylline. It is now generally accepted that the central stimulant effects of caffeine and theophylline are mediated through antagonism of adenosine

receptors in brain (Synder *et al.*, 1981). P_2 purinoceptors preferentially bind nucleotides and are not blocked by methylxanthines.

Biochemical and pharmacological criteria have been used to classify P_1 adenosine receptors into at least two major subtypes, referred to as A_1 and A_2, which either inhibit or stimulate, respectively, the enzyme adenylate cyclase (Van Calker *et al.*, 1979; Londos *et al.*, 1980). The pharmacological profile has shown the agonist rank-order potency at adenosine receptor subtypes as follows:

A_1 receptors: CPA $\geq$ R-PIA > NECA > S-PIA >> CV-1808 > CGS21680.

A_2 receptors: NECA = CGS21680 > CV-1808 > R-PIA > CPA > S-PIA.

For adenosine A_1 receptors there are the highly selective and potent ligands, these include agonists such as CPA, CCPA, CHA and R-PIA and antagonists such as DPCPX and BW-A844U (Lohse *et al.*, 1988; Klotz *et al.*, 1989; Jarvis *et al.*, 1989; Bruns *et al.*, 1987; Patel *et al.*, 1988; Schwabe, 1991). However, ligands selective for adenosine A_2 receptors have been less abundant. CGS21680 and the a-amino-[1,2,4]triazolo-[4,3-a]quinoxaline derivative CP-66713 are the highly A_2-selective agonist and antagonist, respectively (Hutchison *et al.*, 1989; Jarvis *et al.*, 1989; Sarges *et al.*, 1989).

Recently, A_1 and A_2 adenosine receptors have been purified, cloned and expressed in rat brain (Nakata, 1989; Maenhaut *et al.*, 1990; Mahan *et al.*, 1991). The predicted molecular weights of the A_1 receptor and A_2 receptor determined by using molecular cloning techniques are approximately 37 KDa and 45 KDa, respectively, which correspond closely to apparent molecular masses of these

proteins estimated with the purification methods. A_1 and A_2 adenosine receptors are probably small protein members in the G protein-linked receptor superfamily (Linden *et al.*, 1991).

The distribution of A_1 adenosine receptors in rat brain has been well characterized using the radioligand receptor binding both in brain membrane preparations (Murray and Cheney, 1982) and intact slices (Goodman and Snyder, 1982; Weber *et al.*, 1990) and recently even by the use of *in situ* hybridization histochemical analysis (Manhan *et al.*, 1991). High levels of A_1 adenosine receptors are found in the cerebellum, hippocampus and dentate gyrus. High densities also are observed in cerebral cortex, the piriform cortex, the caudate-putamen, the nucleus accumbens. Most white matter areas, as well as certain gray matter areas, such as the hypothalamus, have negligible A_1 receptor concentrations.

The localization of A_2 receptors is markedly different from the regional distribution of A_1 receptors in rat brain. Autoradiographic visualization of A_2 adenosine receptors in rat brain using the selective A_2-selective agonist ligand [^{3}H]CGS21680, indicate that these binding sites are highly localized in the striatum and the olfactory tubercle; no significant amount of specific binding is detected in any other brain regions (Jarvis and Williams, 1989; Parkinson and Fredholm, 1990). The functional role of these A_2 receptors in the brain regions remains unclear. However, these localizations of A_1 and A_2 receptors suggest possible central nervous system sites of action associated with adenosine.

Adenosine Actions in the Central Nervous System

Adenosine as an ubiquitous purine, acting on specific membrane receptors, exerts a wide range of physiological actions throughout the body (Synder, 1985; Stiles, 1986). In the central nervous system, it appears that adenosine serves as a neuromodulator not as a neurotransmitter, since it regulates the activity of numerous other neurotransmitter systems (Synder, 1985). Adenosine and its analogs have been shown to promote inhibition of CNS activity at every level of its organization. The administration of adenosine and adenosine analogs produces sedation (Crawley *et al.*, 1981; Dunwiddie and Worth, 1982; Barraco *et al.*, 1983; Phillis *et al.*, 1986), hypnosis (Marley and Nistico, 1972; Haulica *et al.*, 1973), analgesia (Crawley *et al.*, 1981; Vapaatalo *et al.*, 1975; DeLander and Hopkins, 1987), hypothermia (Vapaatalo *et al.*, 1975), anticonvulsant activity (Dragunow, 1991) and more recently discovered neuroprotective effects (Rudolphi, 1991; Marangos and Miller, 1991) in a variety of animals. These physiological and pharmacological actions of adenosine are probably associated with its powerful neuromodulatory effects that are achieved by a number of mechanisms, the most important of which are its depression of excitatory synaptic transmission and its direct hyperpolarization actions.

It has been shown that adenosine A_1 receptors are located both pre- and postsynaptically. Adenosine A_1 receptors is found on axon terminals of excitatory neurons (Goodman *et al.*, 1983; Dragunow *et al.*, 1988) and also on the dendrites of neurons (Fastbom *et al.*, 1986; Schubert *et al.*, 1985; Tetzlaff *et al.*, 1987).

Presynaptically, adenosine inhibits the release of neurotransmitters such as glutamate, aspartate, and acetylcholine in many brain regions (Dolphin and Archer, 1983; Corradetti *et al.*, 1984; Terrian *et al.*, 1989; Fredholm and Dunwiddie, 1988; Corrieri *et al.*, 1984). This inhibitory effect of adenosine seems to be due to activation of an A_1 receptor linked to a G protein, since pertussis toxin, which inactivates G_i or G_o proteins via ADP ribosylation, blocks the inhibitory effects of adenosine on glutamate release from cultured cerebellar granule cells (Dolphin and Prestwich, 1985). Similarly, N-ethylmaleimide, which also inactivates G proteins linked to the A_1 receptor, blocks adenosine depression of acetylcholine, glutamate and noradrenaline release from hippocampal slices (Fredholm and Lindgren, 1987; Duner-Engstrom and Fredholm, 1988). However, although an inhibitory G protein coupled to A_1 receptors is clearly involved in this presynaptic action of adenosine, adenylate cyclase would appear not to be involved (Fredholm and Lindgren, 1987; Duner-Engstrom and Fredholm, 1988). This presynaptic modulation by A_1 receptors linked via G proteins to both calcium and potassium channels may involve inhibition of calcium influx or stimulation of potassium efflux from the cells (Marangos and Boulenger, 1985; Proctor and Dunwiddie, 1987; Dunwiddie and Fredholm, 1989).

Adenosine, postsynaptically acting on A_1 adenosine receptors, may interact with ion channels and produce hyperpolarization of neurons. Simoes *et al.* (1989) have shown that the adenosine analog CHA inhibits voltage-dependent

sodium channels. Adenosine can inhibit calcium fluxes in a number of systems (Schubert, 1988), and the calcium channel blockade may account for its presynaptic effects on transmitter release and its postsynaptic hyperpolarizing effects. Also, adenosine postsynaptically stabilizes the neuronal membrane potential by opening potassium channels. Adenosine turns on a 4-aminopyridine-sensitive potassium channel (the A current) in hippocampal pyramidal cells (Schubert and Lee, 1986). The postsynaptic actions of adenosine appear to be due mainly to the activation of a G_i protein-coupled potassium channel (Trussell and jackson, 1985).

Both pre- and postsynaptic modulation achieved by activation of A_1 adenosine receptors may contribute to the anticonvulsant and neuroprotective properties of adenosine in the CNS (Dragunow, 1991; Rudophi, 1991). In addition to its important neuromodulatory effects, adenosine acting on A_2 receptors in the cerebrovasculature and on the platelets may increase oxygen and glucose supply by its vasodiatory and antithrombotic effects (Collis, 1989; Born and Cross, 1963). It seems that both A_1 and A_2 adenosine receptors are involved in the neuroprotective effects against ischemia, hypoxia or neurotoxic amino acid-induced brain damage (Rudophi, 1991).

Piriform Cortex

The piriform (olfactory) cortex is a phylogenetically old type of cerebral cortex (Haberly and Price, 1978; Haberly and Bower, 1989). Although the piriform cortex represents the primary receiving area for olfactory sensory input,

its function clearly extends beyond the processing of olfactory information (Haberly and Bower, 1989; Hoffman and Haberly, 1991). The mammalian olfactory cortex has been suggested to be a particular good model system for study of learning and memory due to its neural network architecture and functional organization (Haberly and Bower, 1989). In recent years, several lines of evidence from animals models have suggested that the piriform cortex is highly susceptible to epileptogenesis (Piredda and Gale, 1985; Racine *et al.,* 1988; Honack *et al.,* 1991; Hoffman and Haberly, 1991).

Initially described by Piredda and Gale (1985), an unilateral injection of convulsant drugs such as bicuculline, kainic acid, or carbachol at a specific tiny area within the deep anterior part of the piriform cortex (deep prepiriform cortex) evokes generalized motor seizures at picomole doses that are 20-40 times lower than those required in other parts of the forebrain. Theses authors suggest that this brain area appears to be a crucial epileptogenic site in the rat brain, referred to as *area tempestas* (AT). Recently a locus in the deep posterior part of the piriform cortex has been described as a highly sensitive region responsible for generation of seizure during kindling (Honack *et al.,* 1991). Furthermore, Hoffman and Haberly (1989 and 1991) have reported that induced persistent epileptiform EPSPs (excitatory postsynaptic potentials) in superficial pyramidal cells (Layer II) of the piriform cortex slices are NMDA-dependent processes and this prolonged epileptiform activity may be driven by the deep cells in the endopiriform nucleus, immediately subjacent to the deep piriform cortex (Layer III).

It should be noted in this respect that *area tempestas* described by Piredda and Gale is deep to layer III of the prepiriform cortex (Honack *et al.,* 1991; Hoffman and Haberly, 1991). In the map generated by Piredda and Gale (1985), the injection cannula were placed stereotaxically in the frontal plane and the most sensitive sites are ventrally close to or through the dorsal endopiriform nucleus (DEn). Therefore the convulsion induced by chemicals could have been a consequence of backflow to the dorsal endopiriform nucleus during drug diffusion (Hoffman and Haberly, 1991).

In the beginning of this research project, I established the stereotaxic coordinates to set a "flat brain" orientation and to target the prepiriform cortex (PPC) rather than *area tempestas* in accordance with the atlas of Paxinos and Watson (1982). Using these coordinates, microinjection of bicuculline methiodide (BMI) at a dose of 118 pmol into this region evokes generalized motor seizures (seizure score $\geq$ 4) in more than 90% of animals. The cannula placement in this pharmacologically defined area of the rat PPC is sampled and examined histologically (Fig.I-2). Although the present mapping data are not adequate to determine the extent of the anterior-posterior dimension, it clearly indicates that the sensitive sites are not only more ventrally, laterally placed than that of Piredda and Gale but also the area is not as tiny as they described. These highly sensitive sites to bicuculline methiodide in the PPC described herein also represent the microinjection locus for delivering adenosine related compounds tested in all studies presented in this thesis.

In addition, the DEn is presently tested as an epileptogenic site following focal bicuculline methiodide administration in the study. As illustrated in Fig.I-2, the bilateral motor seizures can be also triggered from this nucleus following unilateral injection of the same dose of bicuculline methiodide. However, the precise relationship and/or comparison between the prepiriform cortex and dorsal endopiriform nucleus involved in initiation and/or generalization of convulsions induced by bicuculline focally injected need to be further investigated.

Objectives

Pharmacological characterization of the adenosine receptor population in the prepiriform cortex and their involvement in the modulation of epileptic seizure activity was presented in this thesis. The adenosine related compounds injected intracerebrally in the PPC as antiepileptic drugs were evaluated in terms of their efficacy, potency and underlying mechanisms against generalized seizures initiated by focal chemoconvulsant challenge. The specific aims associated with this research project are described in details below.

In Chapter II, the efficacy of the adenosine analog NECA against convulsions elicited by kainate, an agonist of one subtype of excitatory amino acid receptors, was examined to understand the neuronal mechanisms underlying the seizure suppressant effects of adenosine analogs in the PPC.

In Chapter III, the ability of the selective A_2 adenosine receptor agonist, CGS21680, to inhibit BMI seizures was determined. In addition, a possible

correlation of the rank order potencies of adenosine analogs as anticonvulsant in the PPC and their affinity for the A_1 adenosine receptor in rat cortical membrane was evaluated. These *in vivo* and *in vitro* investigations further assess the adenosine receptor subtype involved in the observed anticonvulsant effects of adenosine analogs against BMI-induced behavioral seizures.

In Chapter IV, studies were undertaken to evaluate the inhibitory role of endogenous adenosine in the PPC. The anticonvulsant effects of inhibitors of adenosine kinase, adenosine deaminase and nucleoside transport on BMI-induced seizures were pharmacologically characterized. In addition, the proconvulsant properties of adenosine receptor antagonist 8-pSPT was explored using both BMI- and KA-seizure models. The effect of an ecto-5´-nucleotidase inhibitor following focal administration was examined in attempts to further understand the mechanisms of regulation of extracellular adenosine levels and the role of endogenous adenosine as antiepileptic substance in this paleocortical brain area.

In Chapter V, the possibility that activation of adenosine receptors in PPC could inhibit minimal seizure threshold to intravenous bicuculline was tested to delineate the neuroanatomical site for adenosine mediated seizure suppressant actions. Better understanding of adenosine receptor function in this brain area will provide valuable insights into the mechanisms of the inhibitory actions of this purine and the integrative role of this brain area in CNS function.

FIGURE I-1 Adenosine metabolism and transport (modified from Schrader, 1983). SAH: S-adenosylhomocysteine; AdoR: Adenosine receptor.

FIGURE I-1

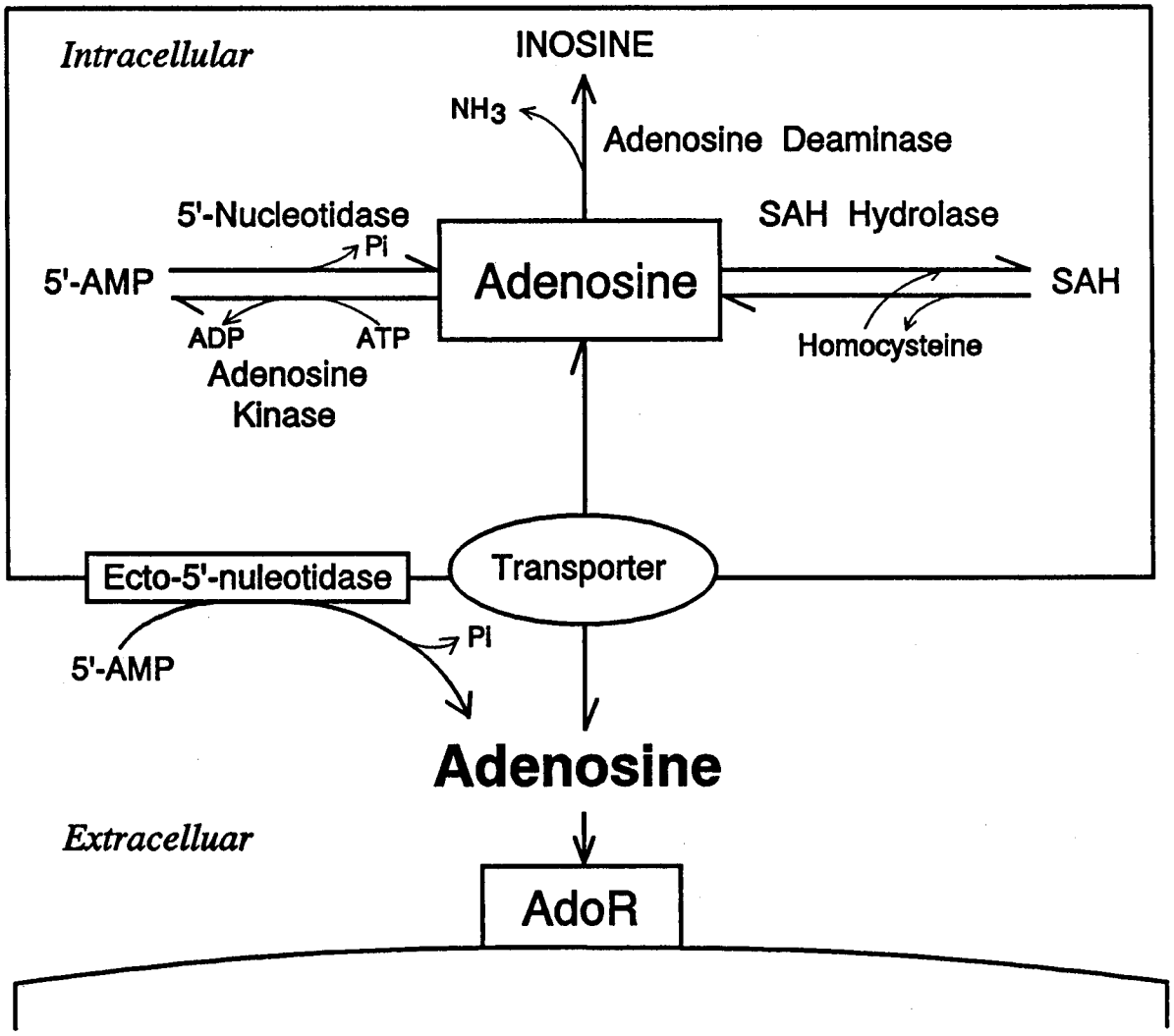
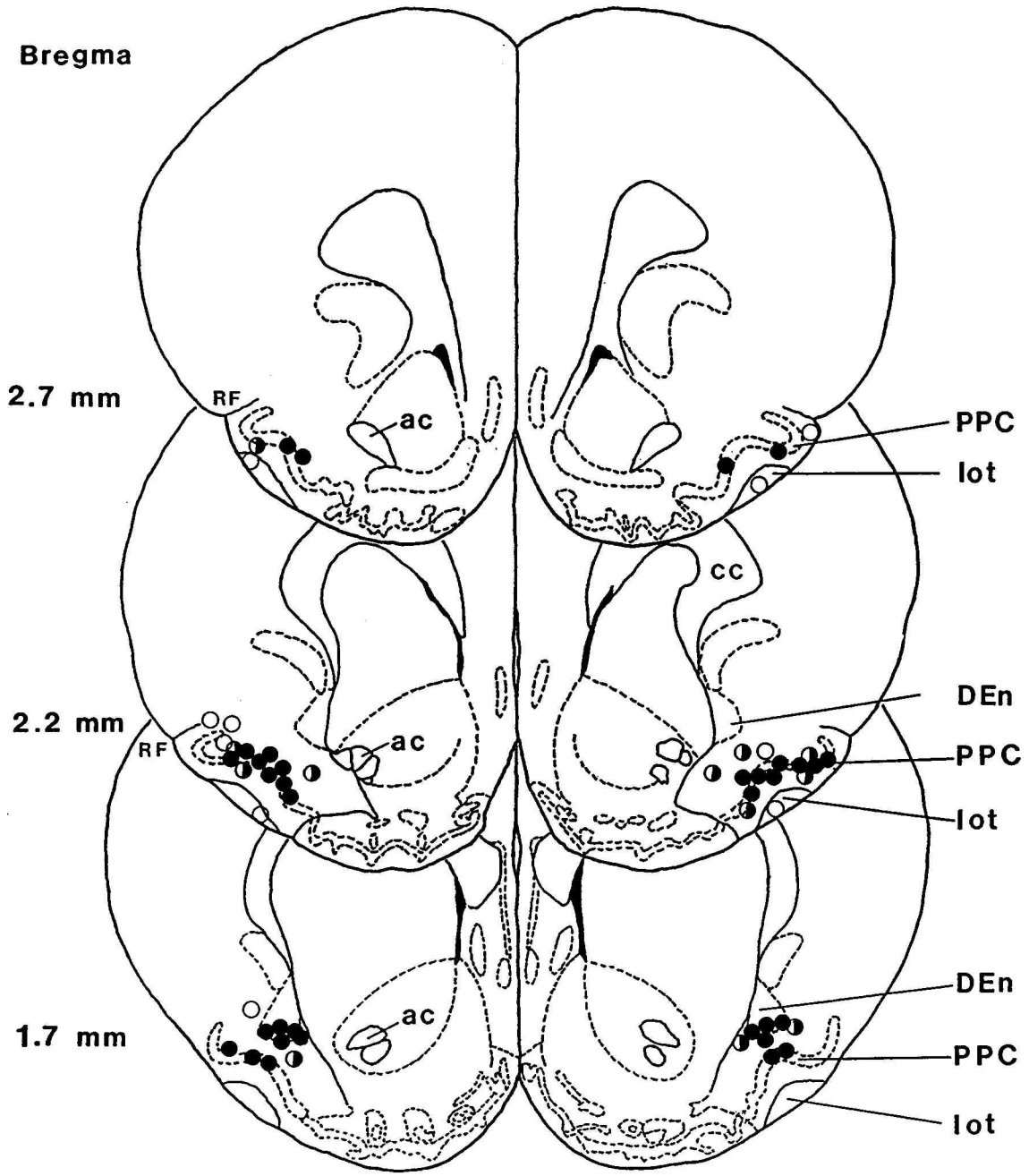

Intracellular
INOSINE
NH3
Adenosine Deaminase
5'-Nucleotidase
Pi
SAH Hydrolase
5'-AMP
Adenosine
SAH
ADP
ATP
Homocysteine
Adenosine
Kinase
Transporter
Ecto-5'-nuleotidase
Pi
5'-AMP
Adenosine
Extracelluar
AdoR

FIGURE I-2 Schematic drawings of coronal sections through rat brain according to the atlas of Paxinos and Watson (1982) showing the unilateral microinjection sites in the prepiriform cortex (PPC) and in the dorsal endopiriform nucleus (DEn). The stereotaxic coordinates are AP 1.8 - 2.1, L 3.3, V - 6.5 for the PPC and AP 1.8, L 3.0, V - 6.0 for DEn when incisor bar is 3.5 mm below the interaural line. The location of injection cannula tips were determined histologically in a series of 16-32 μm coronal sections stained with cresyl violet. Filled circles (●) indicate sites that were the most sensitive for evoking convulsive seizures induced by BMI (score $\geq$ 4). Half-filled circles (◑) represent partial response to BMI (score 2-3). Open circles (○) indicate no or weak response to BMI (score 0-1). Note that plane of the sections shown for histological reference is the same as the plane of the cannula penetration. Abbreviations: ac: anterior commissure; cc: corpus callosum; DEn: dorsal endopiriform nucleus; lot: lateral olfactory cortex; PPC: prepiriform cortex; RF: rhinal fissure.

FIGURE I-2


Bregma
2.7 mm
2.2 mm
1.7 mm
RF
RF
ac
ac
ac
cc
PPC
lot
DEn
PPC
lot
DEn
PPC
lot

CHAPTER II

ANTICONVULSANT EFFECT OF N-ETHYLCARBOXIAMIDOADENOSINE AGAINST KAINIC ACID-INDUCED BEHAVIORAL SEIZURES IN THE RAT PREPIRIFORM CORTEX

Abstract

Kainic acid (KA), microinjected unilaterally into the rat prepiriform cortex (PPC), produces generalized motor seizures in a dose-dependent manner. The adenosine agonist N-ethylcarboxamidoadenosine (NECA), when co-injected with KA, protects against seizures in a dose-dependent and highly potent manner: ED_{50} = 25.6 $\pm$ 2.1 pmol/rat. The seizure-suppressing effects of NECA are completely abolished by co-administration of the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (8-pSPT), suggesting that adenosine receptor activation underlies the efficacy of NECA against KA seizures. Moreover, dilazep, an effective blocker of adenosine uptake, when co-administered with KA, provides significant protection against seizures. Together, these findings suggest that adenosine receptors may play an important role in the regulation of the inhibitory neuronal circuitry of this paleocortical brain area.

Abbreviations: KA, kainic acid; NECA, N-ethylcarboxamidoadenosine; 8-pSPT, 8-(p-sulfophenyl)theophylline; PPC, prepiriform cortex.

Introduction

Although considerable experimental evidence suggests that endogenous adenosine may function as an inhibitory modulator of epileptogenesis (Barraco *et al.*, 1984; Dunwiddie and Worth, 1982; Eldridge *et al.*, 1989; Maitre *et al.*, 1974; Murray and Szot, 1986; Szot *et al.*, 1987; Winn *et al.*, 1980), the neuroanatomical and neurochemical basis for the anticonvulsant actions of adenosine have not been established. Recently, we have shown that focal activation of adenosine receptors in the prepiriform cortex (PPC), a brain region which may play a significant role in seizure initiation and propagation (Piredda and Gale, 1985) protects rats from bicuculline-induced seizures (Franklin *et al.*, 1988; Franklin *et al.*, 1989). This report was the first to specifically identify an adenosine receptor population with the capacity to suppress epileptic seizures. In order to understand more fully the neuronal mechanism(s) subserving this response, we evaluated the efficacy of N-ethylcarboxamidoadenosine (NECA) against seizures induced by kainic acid (KA), a convulsant which induces seizures through a mechanism independent from that of the τ-aminobutyric acid (GABA) receptor antagonist bicuculline.

Materials and Methods

Male Sprague-Dawley rats (300-400 g) maintained at 22 °C on a standard 12 hour light/dark schedule, with ad libitum access to food and water were anaesthetized with Equithesin (2.7 ml/kg, i.p.) for stereotaxic surgery. With the incisor bar lowered 3.5 mm below the interaural line each animal was implanted unilaterally with paired stainless-steel 22 gauge guide, and 28 gauge injection cannulas directed to a site in PPC 6.5 mm below dura and 3.3 mm lateral, and 1.8-2.1 mm anterior to bregma. Injection cannulas always extended at least 1.5 mm beyond the guide cannula terminus. Animals were allowed at least 24 hour recovery from surgery before experimentation, and each animal received no more than a single set of experimental injections which were separated by a minimum interval of 24 hours. All experiments were carried out during the 12 hour light cycle.

Intracerebral microinjections were performed as described previously (Franklin *et al.,* 1989). Kainic acid and all drugs were dissolved in normal saline and co-injected at a rate of 0.9 nl/sec. in a total volume of 120 nl. Severity of generalized motor seizures induced by either bicuculline methiodide (BMI) or KA was scored as follows: 0, no seizure; 1, myoclonic jerks of the contralateral forelimb; 2, mild forelimb clonus (± mouth and facial movements-clonus of jaw and vibrissae and head nodding) lasting more than 5 sec.; 3, severe forelimb clonus lasting more than 15 sec.; 4, rearing in addition to forelimb clonus; 5, loss of balance and/or falling in addition to rearing and forelimb clonus. On day 1

animals were challenged with a dose of 118 pmol BMI injected into the PC, and only rats with seizure scores of 4 or 5 were used for subsequent studies. Focal injection of KA alone or co-injection of this compound with NECA, with NECA + 8-(*p*-sulfophenyl)theophylline (8-pSPT) or dilazep followed on day 2. If animals exhibited a reduced seizure score on day 2, an anticonvulsant effect of drug treatment was confirmed by a post-test with BMI on day 3. Animals not displaying the same sensitivity to BMI as on day 1 were excluded from data analysis.

Results

In order to determine a challenging dose of KA, we injected doses of 100, 150 and 200 pmol KA in the PPC. As shown in Table I-1, KA treatment resulted in a dose-dependent production of bilateral generalized motor seizures. A dose of 200 pmol/rat reached the ED_{100} criterion of seizure score ≥ 4. To determine the anticonvulsant efficacy of adenosine analogs against KA-induced seizures in the PPC, various doses of NECA were co-administered with a 200 pmol dose of KA. Behavioral seizures elicited by KA were potently suppressed by NECA in a dose-dependent manner (Table II-2). NECA at doses $\geq$ 40.5 pmol significantly reduced the mean seizure scores as compared to KA alone. Fitting the NECA dose-response data to a four-parameter logistic equation by non-linear, least squares regression analysis employing an iterative method (FITFUN, public procedure of the NIH-PROPHET data analysis system) of residual minimization, revealed that NECA suppressed KA seizures with an ED_{50} value of 25.6 $\pm$ 2.1 pmol/rat.

As shown in Fig.II-1, the anticonvulsant effects of administration of 81 pmol NECA, a dose which effects a 83 % reduction in mean seizure score against KA, were completely reversed by co-administration of the specific adenosine receptor antagonist 8-pSPT (1.61 nmol). Inasmuch as 8-pSPT administered at this dose level in the absence of NECA had no effect on KA-induced seizures (Fig.II-1), it is apparent that the anticonvulsant effects of NECA against KA-induced seizures in the PPC are mediated through adenosine

receptor activation. Further support for an adenosine receptor-mediated basis for the anticonvulsant effects of NECA was indicated by the significant protection provided by dilazep against KA-induced seizures in this brain area (Table II-2). Dilazep, which potentiates the effects of endogenous adenosine through inhibition of the nucleoside transporter (Williams *et al.*, 1984), when co-administered at a dose of 49.6 nmol with KA (200 pmol) provide a 79 % reduction in mean seizure score from the control response to KA alone (Table II-2).

Discussion

In this study we have shown that the stable adenosine analogue, N-ethylcarboxamidoadenosine (NECA), inhibits KA-elicited seizures in a dose-dependent manner in the PPC. The anticonvulsant activity of NECA was antagonized by 8-pSPT, a selective adenosine receptor antagonist. These results suggest that focal activation of adenosine receptors in the PPC underlies the anticonvulsant action of NECA, and further support our hypothesis that adenosine receptors in the PPC play a fundamental role in the modulation of seizure susceptibility. The importance of the inhibitory role of endogenous adenosine is underscored by the potent and efficacious protection against KA-induced seizures provided by dilazep.

KA has been shown to stimulate excitatory amino acid release through activation of presynaptic receptors in the hippocampus and cerebellum, as well as in the primary olfactory cortex (Collins *et al.*, 1983; Ferkany *et al.*, 1982). KA-induced seizures in the PPC could, therefore, result either from such a presynaptic process or through a direct postsynaptic action at KA receptors. The anticonvulsant activity of adenosine and adenosine analogs in the PPC may be the result of either pre- or postsynaptic processes, as well. Adenosine has been shown to exert its inhibitory influence both by direct reduction of postsynaptic excitatory potentials and also by inhibition of excitatory neurotransmitter release (Dolphin and Archer, 1983; Fredholm and Dunwiddie, 1988).

Several lines of evidence have linked adenosine receptor activation to the

regulation of excitatory amino acid release. Adenosine receptors have been localized on the axon terminals of excitatory neurons (Goodman *et al.*, 1983) and activation of adenosine receptors has been shown to decrease aspartate and glutamate release in many brain areas (Dolphin and Archer, 1983; Motley and Collins, 1983). In addition, it has been reported recently that the metabolically stable adenosine analog 2-chloroadenosine protects rats from KA-induced neurotoxicity in the striatum (Arvin *et al.*, 1988).

The seizure suppressing effects of NECA against KA in the PPC described in this report are not inconsistent with a presynaptic mechanism of adenosine action whereby adenosine receptors inhibit excitatory neurotransmitter release in the domain of output neuron of the PPC; however, the contribution of a direct postsynaptic site of action cannot be excluded by these data. The sensitivity of KA-induced seizures to suppression by adenosine receptor activation which we have described herein, considered together with our previous report describing the suppression of BMI-induced seizures in the PPC (Franklin *et al.*, 1988; Franklin *et al.*, 1989), suggests that adenosine receptors may either directly regulate the activity of the excitatory output neurons of this brain region or, at a minimum, regulate a locus of convergence of two separate excitatory influences in the PPC. This paleocortical area clearly represents a significant central nervous system locus for the anticonvulsant effects of adenosine analogs. Further investigations are warranted to elucidate the mechanisms by which adenosine receptors exert an inhibitory modulation in this region of the forebrain.

Acknowledgement

This work was supported by USPHS grant NS-23227 to T.F.M.

TABLE II-1

Convulsant effects of kainic acid (KA) in the rat prepiriform cortex

Kainic Acid (pmol)	Distribution of Seizure Scores 0	1	2	3	4	5	Mean Seizure Score (n)
100	3				1	1	1.80 (5)
150			1		1	1	3.67 (3)
200					3	10	4.77 (13)

Kainic acid was microinjected at the indicated doses unilaterally into the prepiriform cortex as described in the text. Animals were then observed for a 120 min epoch and the mean highest seizure score attained for each group of animals of size (n) for each treatment was determined. The distribution of seizure scores of animals within a treatment are tailed under the bold numerical columns corresponding to each level of seizure severity as defined in the text.

TABLE II-2

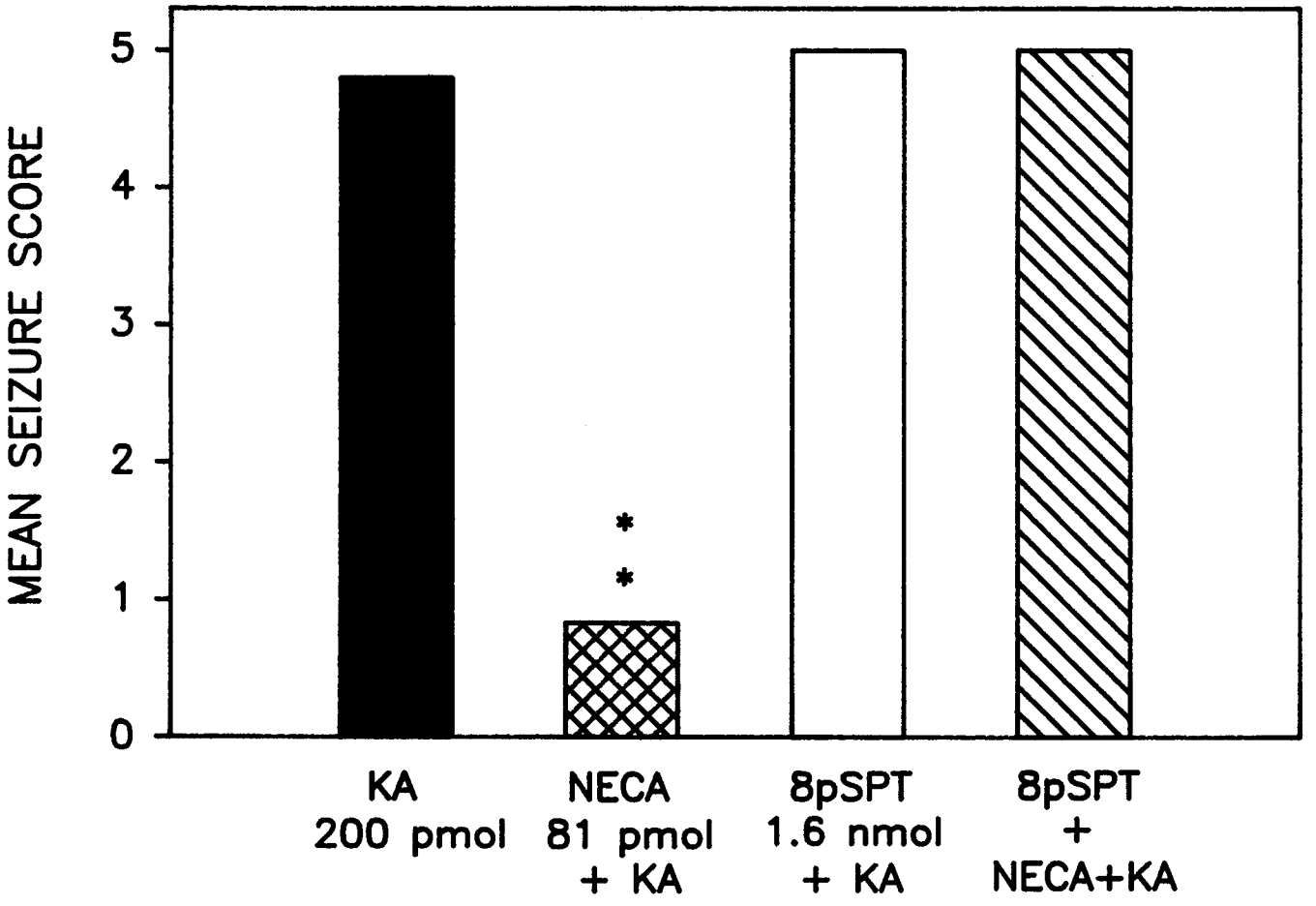
Anticonvulsant effect of NECA and dilazep against kainic acid-induced seizures in the rat prepiriform cortex

Treatment Kainic Acid: 200 pmol + DRUG: DOSE		Distribution of Seizure Scores 0	1	2	3	4	5	Mean Seizure Score (n)	Percent Protection
NECA: (pmol)	0					3	10	4.77 (13)	—
	16					3	1	4.25 (4)	10.9
	40.5	2	1		1			1.00 (4)	79.0**
	81	5					1	0.83 (6)	82.6**
	162	3	1					0.25 (4)	94.8**
Dilazep: (nmol)	49.6	4					1	1.00 (5)	79.0**

Kainic acid (200 pmol) was co-injected with NECA or dilazep at the indicated doses unilaterally into the prepiriform cortex as described in the method. Animals were then observed for a 120 min epoch and the mean highest seizure score attained for each group of animals of size(n) for each treatment was determined. The distribution of seizure scores of animals within a treatment are tailed under the bold numerical columns corresponding to each level of seizure severity as defined in the text. Percent protection reflects the reduction in mean seizure stage from mean control response to 200 pmol kainic acid.
** Statistically lower ($p < 0.01$, one tailed rank-sum test) than control KA (200 pmol) response.

FIGURE II-1 Reversal of the protective effects of NECA against kainic acid (KA)-induced epileptic seizures by the specific adenosine receptor antagonist 8-pSPT. KA, KA + NECA, KA + 8-pSPT or KA + NECA + 8-pSPT at the indicated doses were microinjected unilaterally into the PPC as described in the text and animals were then each observed for an interval of 120 min. Values represent the mean highest seizure scores attained by animals (n=4-13) from each treatment class. ** Significantly reduced mean seizure score from mean seizure score of kainic acid alone ($p < 0.01$, one tailed rank-sum test).

FIGURE II-1


MEAN SEIZURE SCORE
5
4
3
2
1
0
*
*
KA
200 pmol
NECA
81 pmol
+ KA
8pSPT
1.6 nmol
+ KA
8pSPT
+
NECA+KA

CHAPTER III

ANTICONVULSANT EFFECT OF CGS21680 MEDIATED BY A_1 ADENOSINE RECEPTORS

Abstract

Following focal injection of adenosine analogs against bicuculline methiodide-induced generalized seizures in the rat prepiriform cortex, CGS21680, an A_2-selective adenosine agonist, was the least potent anticonvulsant (ED_{50} = 605.2±46.7 pmol/rat). Pharmacological characterization of this response in this brain area revealed a significant correlation ($r=0.85$, $p < 0.01$) between the anticonvulsant potency of eight adenosine analogs and their respective affinities for the A_1 adenosine receptor. These results suggest that activation of adenosine A_1 receptors underlies the anticonvulsant action of adenosine analogs.

Abbreviations: BMI, bicuculline methiodide, CGS21680, 2-[p-(2-carboxyethyl)-phenylamino]-5'-N-ethylcarboxamidoadenosine; CHA, cyclohexyladenosine; 2-ClA, 2-chloroadenosine; CPA, cyclopentyladenosine; CV-1808, 2-phenylaminoadenosine; [^{3}H]DPCPX, 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine; NECA, N-ethylcarboxamidoadenosine; R- and S-PIA, R- and S-phenylisopropyladenosine; PPC, prepiriform cortex.

Introduction

A number of experiments have been performed to investigate the anatomical sites and neurochemical mechanisms for the anticonvulsant actions of adenosine in the central nervous system (CNS) since adenosine was reported to be involved in seizure expression over a decade ago. The results of our previous studies have demonstrated that adenosine receptor population in the rat prepiriform cortex (PPC) plays an fundamental role in suppression of both bicuculline methiodide (BMI) and kainic acid initiated seizures (Franklin et., 1988; Franklin et al., 1989; Zhang et al, 1990). The seizure suppressant responses of adenosine are believed to be mediated via an interaction with A_1 adenosine receptor subtype mainly based on pharmacological profiles *in vivo* (Franklin et al., 1989). However, argument continues regarding whether A_1 or A_2 subtypes of adenosine receptors mediate the inhibitory function in the CNS. The availability of CGS21680, the first agonist with high affinity and selectivity for A_2 adenosine receptors, would aid in the resolution of this problem. In the present paper, we first examined the efficacy and potency of CGS21680 as an anticonvulsant against BMI seizures in PPC. Secondly, we performed radioligand competition experiments using CGS21680 and seven other adenosine agonists as displacers of 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX) binding in rat brain. Therefore, a possible correlation between the potencies of eight adenosine agonists as seizure suppressants *in vivo* and their affinities for adenosine A_1 receptors *in vitro* was explored in this brain region.

Materials and Methods

Materials

2-[p-(2-Carboxyethyl)-phenylamino]-5'-N-ethylcarboxamidoadenosine sodium salt (CGS21680C) was a generous gift from CIBY-Geigy Corporation (Summit, NJ). R- and S-phenylisopropyladenosine (R- and S-PIA), N-ethylcarboxamidoadenosine (NECA) and adenosine deaminase were purchased from Boehringer-Mannheim (Mannheim, West Germany). Cyclopentyladenosine (CPA), 2-phenylaminoadenosine (CV-1808) and bicuculline methiodide (BMI) were obtained from Research Biochemicals, Inc. (Wayland, MA). 2-Chloroadenosine (2-ClA) and GTP were from Sigma Chemical Company (St. Louis, MO). Cyclohexyladenosine (CHA) was obtained from Calbiochem (La Jolla, CA). [^{3}H]DPCPX (120 Ci/mmol) was purchased from DuPont NEN (Boston, MA).

Behavioral assay

Male Sprague-Dawley rats (320-400 g) were maintained at 21 $\pm$ 1°C with a standard 12/12 hour light/dark cycle. Stereotaxic surgery, microinjection technique and drug treatment protocol were performed using methods described in detail by Franklin et al. (1989). Animals were anaesthetized with Equithesin and unilaterally implanted with paired 22-gauge guide, and 28-gauge injection cannulas into the right prepiriform cortex. Stereotaxic coordinates for the injection site within the prepiriform cortex were: 1.8-2.1 mm anterior to bregma, 3.3 mm lateral and 6.5 mm below dura when the incisor bars were set at 3.2-3.5

mm below the interaural line. All compound solutions used in this study were prepared in saline and unilaterally injected in the PPC in a volume of 120 nl at a rate of 0.9 nl/s. Following surgery animals were allowed a 1- to 2-day recovery period, then were first challenged by 118 pmol of BMI after saline vehicle pre-injection (15 min) on day 1, and those animals with seizure score $\geq$ 4 were used in subsequent studies. On day 2, the adenosine analog CGS21680 was pre-treated (15 min) prior to BMI challenge in the PPC. If animals exhibited reduced seizure scores on day 2, 118 pmol of BMI was given to them in post-test on day 3. Those animals that displayed reduced sensitivity to BMI in the post-test were excluded from studies and their cannula placements were histologically examined. After single injection of the convulsant bicuculline methiodide in the PPC, generalized seizure behavior was quantified according to a scoring system ranged from seizure score 0 to 5 with increasing seizure severity as previously described (Racine, 1972). All testings of behavioral seizure activity were performed on conscious unrestrained animals during the light cycle and separated by an interval of 20-28 hours.

Membrane preparation and binding assay

The fresh frontal, ventral parts of cerebral cortex from a male Sprague-Dawley rat were homogenized in 10 mM Tris buffer (pH 7.7) containing 10 mM EDTA and centrifuged at 37,000 g at 4°C for 10 min. The pellet was resuspened in 50 mM Tris buffer with 1 mM EDTA and centrifuged as described above. The resuspension was incubated in the same buffer containing 7.5 IU/ml

adenosine deaminase, 150 mM NaCl and 100 μM GTP at 37°C for 30 min . Following centrifugation as above, the pellet was washed three times in 50 mM Tris buffer, and the final pellet was resuspened in a 50 mM Tris buffer with 2.5 mM $MgCl_2$. The binding assay was performed using a modification of the method described previously (Leid et al., 1988). [^{3}H]DPCPX, is a high affinity antagonist radioligand which selectively labels A_1 adenosine receptors (Bruns et al., 1987), and adenosine agonists CPA, CHA, R-PIA, S-PIA, NECA, 2-ClA, CV-1808 and CGS21680 as displacers were used in the competition experiments. All adenosine agonists were prepared in distilled water except R-PIA. R-PIA was initially dissolved in 5% volume of 0.1N HCl, then diluted with distilled water and neutralized in 1% volume of 0.5 N NaOH to 1 mM stock concentration. The order of additions was displacer or vehicle (5 μl), membranes (125 μl, 60-100 μg protein), 50 mM Tris buffer (pH 7.7) containing 2.5 mM $MgCl_2$ (845 μl) and [^{3}H]DPCPX (25 μl). Binding reactions were carried out in 50 mM Tris buffer with 2.2 mM $MgCl_2$ using a final [^{3}H]DPCPX concentration of 0.1 nM in a volume of 1 ml at 22°C for 90 min. Incubations were terminated by rapid filtration over GF/B filters using a Brandel Cell Harvester (Model M-24R, Brabdel Instruments, Gaithersburg, MD), followed by rapid washing of the filters four times with 4 ml ice-cold Tris buffer. Filter strips were presoaked in 0.5% polyethyleneimine (Sigma) to reduce non-specific binding. Nonspecific binding was defined in the presence of 100 μM 2-ClA. Filter disks were allowed to elute overnight in 4 ml Biocount Scintillation cocktail (Research Products

International Corp., Mount Prospect, IL) and then counted using a Beckman LS6800 scintillation counter at an efficiency of approximately 50%. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as the standard.

Results

Focal injection of CGS21680 elicited a dose-dependent protection against BMI induced seizures with an ED_{50}±S.D. of 605.2 ± 46.7 pmol/rat (Fig.III-1). Among all adenosine analogs examined, CGS21680 was the least potent compound after unilateral focal injection. CGS21680 was approximately from 30- to 70-fold less potent than selective A_1 adenosine analogs of CHA, CPA and R-PIA.

For all equilibrium competition curves of adenosine agonists (Fig.III-2), a two-site model significantly improved the fit, when compared with the fit of the data to a one-site model (data not shown). IC_{50} values from high affinity sites which represented adenosine agonist- bound A_1 receptors interacting with G-protein to form a ternary complex were used in subsequent correlation study. Agonist rank order potency of inhibiting the specific binding of [^{3}H]DPCPX was CPA ≥ CHA = R-PIA > NECA > 2-ClA > S-PIA >> CV-1808 >> CGS21680. Linear regression analysis was performed on inhibition of [^{3}H]DPCPX binding by adenosine analogs and their anticonvulsant potency, as depicted in Fig.III-3. The rank order potency of adenosine analogs *in vivo* was significantly ($p < 0.01$) related to that of their respective IC_{50} values as inhibitors of [^{3}H]DPCPX binding *in vitro* with a correlation coefficient (r) value of 0.85.

Discussion

The most important finding in this study is that the rank order of potency as anticonvulsants *in vivo* closely parallels that of the abilities of these adenosine analogs to inhibit [^{3}H]DPCPX binding *in vitro*. This significant relationship between the potencies of these compounds in interaction with A_1 adenosine receptor and suppression of seizure behavior suggests that A_1 subtype of adenosine receptors may effect the observed inhibition of bicuculline-induced seizures in the PPC. In the previous study we have shown that enhanced *in vivo* potency of NECA as an anticonvulsant was largely influenced by the distribution and disposition of this compound in brain following intracerebral administrations (Franklin et al., 1989).

CGS21680 has been introduced as a selective and potent A_2 adenosine agonist with an approximately 140 fold selectivity for A_2 over A_1 receptors in rat brain (Hutchinson et al., 1989). CGS21680 has been shown to depress the cerebral neuronal firing using iontophoresis to deliver drug solution in rat brain (Phillis, 1990). However, the author interpreted that this inhibitory response of CGS21680 was mediated through A_2 adenosine receptors so that A_2 adenosine receptor was also involved in the regulation of neuronal excitability in cerebral cortex. This conclusion was mainly based on that CGS21680 as a selective A_2 agonist effectively elicited depressant response in cerebral cortical neurons. Under Phillis's (1990) experimental conditions it impossible to make any potency comparison among adenosine analogs in terms of their effective concentrations

using iontophoresis even though he reported that CGS21680 was equipotent with adenosine. In the present study, CGS21680 protected rats against bicuculline-induced seizures in the PPC like other adenosine analogs but with the least potency. Consistent with the present observation, Lupica et al. (1990) have indicated that giving higher concentration of CGS21680 than that of A_1 adenosine agonists is able to inhibit the excitatory postsynaptic potential (EPSP) in rat hippocampal slice. They (Lupica et al. , 1990) also demonstrated that the EC_{50} value of CGS21680 at inhibiting EPSP is similar to that for adenosine and is far below the affinity of CGS21680 for A_2 receptors in striatal tissue (Hutchinson et al., 1989). Low potency of CGS21680 in the electrophysiological responses in hippocampal slice and in the anticonvulsant studies in the prepiriform cortex were consistent with its low affinity for A_1 receptors. These findings also represent strong evidence that CGS21680 at high concentrations not only binds to A_1 adenosine receptors but also is an agonist at these receptors.

In summary, that A_1 adenosine receptors underlie the anticonvulsant effects of CGS21680 was strongly supported by the following findings: (1) CGS21680, a selective A_2 adenosine agonist, was a much less potent compound as compared to A_1 adenosine analogs; (2) the rank order potency of eight adenosine analogs as anticonvulsants significantly correlated with their affinity for A_1 adenosine receptors; (3) the piriform cortex as one primary part of cerebral cortex had a relatively higher density of A_1 adenosine receptors

(Goodman and Synder, 1982), whereas this brain region lacked specific binding of [^{3}H]CGS21680 using this A_2 selective radioligand in the recent autoradiographic study (Jarvis and Williams, 1989; Parlinson and Fredholm, 1990). The seizure suppressant response of adenosine analogs therefore appears to be mediated through A_1 subtype of adenosine receptors in the rat prepiriform cortex.

Acknowledgement

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FIGURE III-1 The dose-response curve for the protective effects of CGS21680 on bicuculline methiodide (BMI)-induced convulsions in PPC. Data points represent the percent reduction of mean seizure score of CGS21680 pretreatment from mean control response to BMI alone (118 pmol) from groups of animals (n=4). ED_{50} value $\pm$ S.D. of CGS21680 was estimated using an nonlinear least-squares regression line fitted to the four-parameter logistic equation with the Prophet computer system procedure FITFUN.

FIGURE III-1

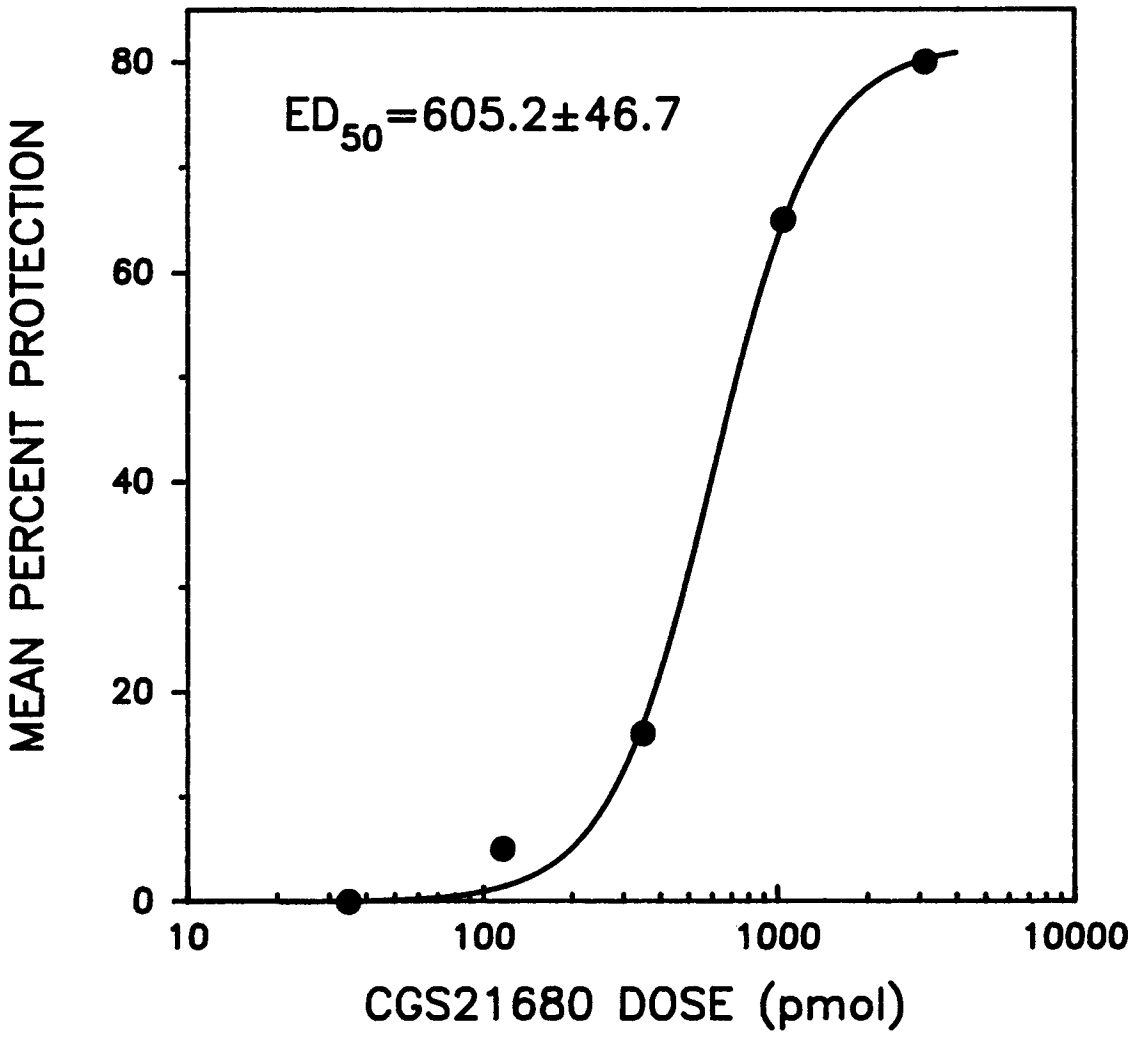

ED50=605.2±46.7
MEAN PERCENT PROTECTION
80
60
40
20
0
10
100
1000
10000
CGS21680 DOSE (pmol)

FIGURE III-2 Agonist competition of [^{3}H]DPCPX binding in a frontal cortical membrane preparation of rat brain. Curves drawn are based on theoretical two-site parameter estimates determined by nonlinear regression analysis using the Prophet FITCOMP Procedure.

FIGURE III-2

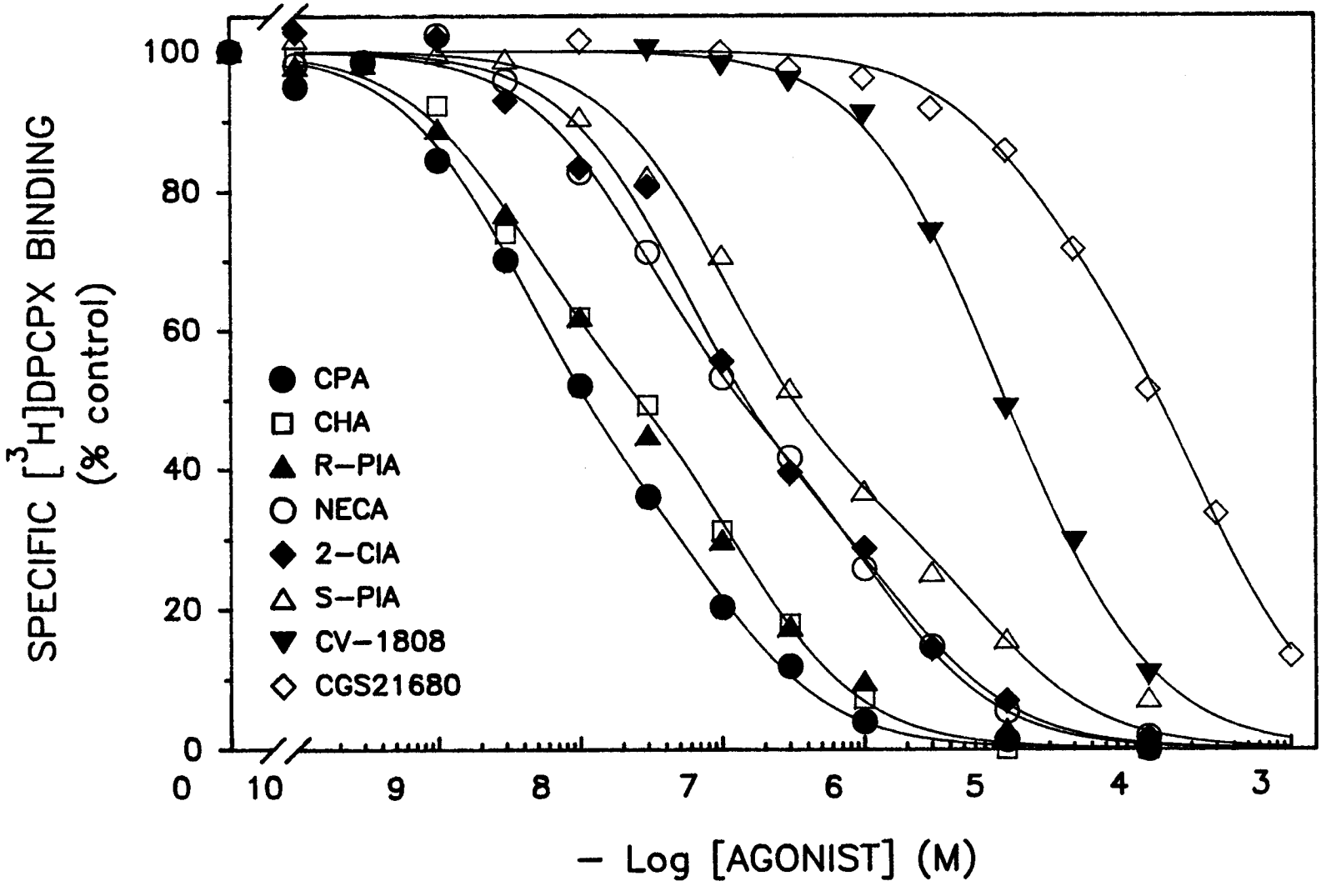
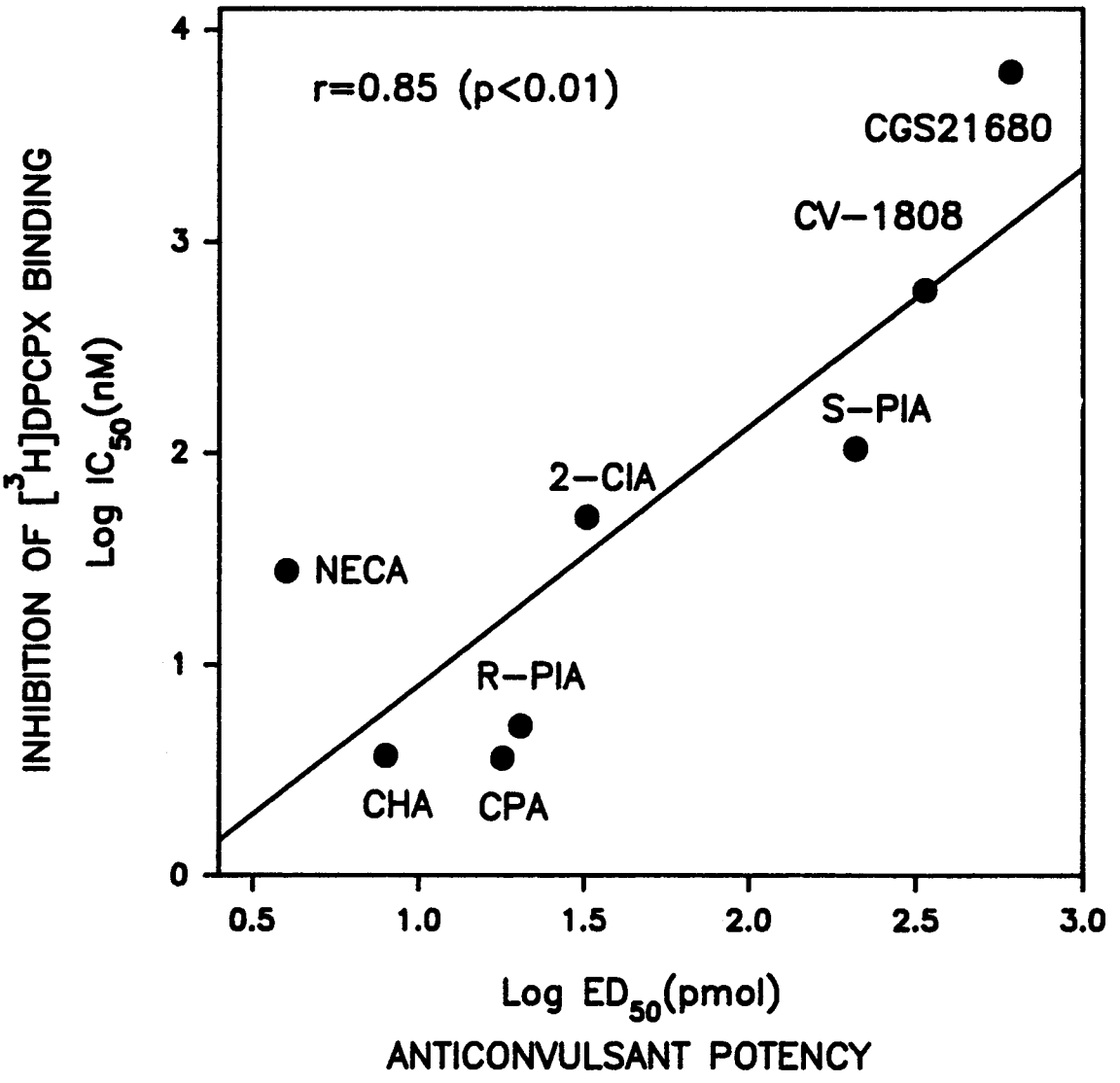

SPECIFIC [3H]DPCPX BINDING
(% control)
100
80
60
40
20
0
0
10
9
8
7
6
5
4
3
CPA
CHA
R-PIA
NECA
2-CIA
S-PIA
CV-1808
CGS21680
- Log [AGONIST] (M)

FIGURE III-3 Correlation between adenosine agonists as anticonvulsants and their affinity for A_1 adenosine receptors in rat brain. ED_{50} data of other seven adenosine analogs were from our previous study (Franklin et. al., 1989). ED_{50} value of CV-1808 was arbitrarily set at the highest inactive dose of 335 pmol. [^{3}H]DPCPX was used to label A_1 receptors in the competition experiments. IC_{50} values of adenosine analogs as inhibitors at high affinity sites of [^{3}H]DPCPX specific binding were determined by nonlinear regression analysis using the Prophet FITCOMP Procedure. The regression line and correlation coefficient (r) were derived by use of linear regression program in GraphPAD software (GraphPAD, San Diego, CA).

FIGURE III-3


r=0.85 (p<0.01)
CGS21680
CV-1808
S-PIA
2-CIA
NECA
R-PIA
CHA
CPA
INHIBITION OF [3H]DPCPX BINDING
Log IC50(nM)
Log ED50(pmol)
ANTICONVULSANT POTENCY
0
1
2
3
4
0.5
1.0
1.5
2.0
2.5
3.0

CHAPTER IV

MANIPULATION OF ENDOGENOUS ADENOSINE IN THE RAT PREPIRIFORM CORTEX MODULATES SEIZURE SUSCEPTIBILITY

Abstract

Our previous studies suggest that A_1 adenosine receptors in rat prepiriform cortex (PPC) play an important role in the inhibition of bicuculline methiodide (BMI) or kainic acid (KA)-induced convulsions. In the present study, we evaluated manipulation of endogenous adenosine in this brain area as a strategy to effect seizure suppression. All compounds evaluated were unilaterally microinjected into the PPC. Administration of exogenous adenosine afforded a dose-dependent protection (ED_{50} = 48.1±8.4 nmol) against BMI-induced seizures, and these anticonvulsant effects were significantly potentiated by treatment with an adenosine kinase inhibitor, 5′-amino-5′-deoxyadenosine (5′$NH_2$5′dADO); by the adenosine transport blockers, dilazep or nitrobenzylthioinosine 5′-monophosphate; and by an adenosine deaminase inhibitor, 2′-deoxycoformycin (2′DCF). When administered alone, 5′$NH_2$5′dADO, 5′-iodotubercidin and dilazep were found to be highly efficacious as anticonvulsants with respective ED_{50} values of 2.6±0.8, 4.0±2.7 and 5.6±1.6 nmol. In contrast, 2′DCF was both less potent and less efficacious. Theses results suggest that accumulation of endogenous adenosine may contribute to seizure suppression, and that adenosine kinase and adenosine transport system may play a pivotal role in the regulation of extracellular levels of adenosine in the CNS. The adenosine antagonist, 8-(*p*-sulfophenyl)theophylline, markedly increased the severity of both BMI- and KA-induced seizures. Moreover, reduction of extracellular adenosine formation by a

focal injection of an ecto-5´-nucleotidase inhibitor, α, β-methyleneadenosine diphosphate, produced generalized seizures (ED_{50}=37.3$\pm$22.7 nmol). Together the proconvulsant effect of an adenosine receptor antagonist and the convulsant action of ecto-5´-nucleotidase inhibitor further support the role of endogenous adenosine as a tonically active antiepileptogenic substance in the PPC.

Abbreviations: AICAr, 5-amino-4-imidazolecarboxamide riboside; AOPCP, α, β-methylene adenosine diphosphate; A_1R, adenosine A_1 receptor; BMI, (-)-bicuculline methiodide; CNS, central nervous system; 2´DCF, 2´-deoxycoformycin; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; KA, kainic acid; NBPMR, nitrobenzylthioinosine (6-(4-nitrobenzylmercapto)purine ribonucleoside); NBPMR-PO_4, nitrobenzylthioinosine 5´-monophosphate, 5´$NH_2$5´dADO, 5´-amino-5´-deoxyadenosine; PPC, prepiriform cortex 8-*p*SPT, 8-(*p*-sulfophenyl)theophylline; SAH, S-adenosylhomocysteine.

Introduction

Adenosine is an endogenous purine nucleoside which exerts characteristic inhibitory actions on neuronal firing rates, synaptic transmission and neurotransmitter release in the central nervous system (Phillis and Wu, 1981; Snyder, 1985). It has been shown that adenosine functions as a modulator particularly within the cardiovascular and nervous system by acting on extracellularly directed adenosine receptors which are classified into at least two categories, termed A_1 (R_a) and A_2 (R_i) (Van Calker *et al.,* 1979; Londos *et al.,* 1980). A_1 and A_2 adenosine receptors were initially characterized on the basis of their ability to either inhibit or activate the enzyme adenylate cyclase, respectively. More recently A_1 and A_2 adenosine receptors have been distinguished with respect to their agonist pharmacological profiles. Both A_1 and A_2 receptor mediated effects are blocked by the methylxanthines caffeine and theophylline which act as competitive adenosine receptor antagonists (Sattin and Rall, 1970).

There is considerable evidence to indicate that adenosine and adenosine analogs possess anticonvulsant properties, presumably as a consequence of inhibition of seizure initiation and propagation (Dragunow, 1991). The anticonvulsant action of adenosine was initially described by Maitre and co-workers in 1974 who found adenosine blocked audiogenic seizures in mice (Maitre *et al.*, 1974). Subsequent studies using adenosine and adenosine analogs have shown that these compounds inhibit various chemoconvulsant-induced

motor seizures (Snyder *et al.*, 1981; Dunwiddie and Worth, 1982; Murray *et al.*, 1985) and kindled seizures (Albertson *et al.*, 1983; Barroco *et al.*, 1984; Dragunow *et al.*, 1985). Interestingly, it was found that adenosine levels in the brain were increased 3-5 fold during bicuculline induced seizure activity (Winn *et al.*, 1980 and Schrader *et al.*, 1980). Moreover, it has been demonstrated that methylxanthines such as caffeine, theophylline and aminophylline exert convulsant (Braude and Krantz, 1965; Zwillich *et al.*, 1975; Persson and Erjefalt; 1981; Chu, 1981) or proconvulsant effects (Albertson *et al.*, 1983; Murray *et al.*, 1985; Ault *et al.*, 1987; Eldridge *et al.*, 1989) in a wide range of experimental models. These data not only suggest that adenosine receptor-coupled processes may be involved in the etiology of certain epileptic phenomena, but also indicate that the excitatory state of neurons in the CNS is regulated by the homeostatic influence of endogenous adenosine. Most *in vivo* studies which have investigated adenosine involvement in the expression of seizures, however, have employed systemic or intracerebroventricular (i.c.v.) injections of adenosine analogs; thus neither the neuroanatomic sites nor the mechanism(s) for the anticonvulsant actions of adenosine were addressed. Furthermore, because of their potent peripheral effects, *in vivo* studies with systemically administered adenosine and adenosine analogs must be interpreted cautiously; even i.c.v. administration of adenosine analogs produces hypotensive, sedative and myasthenic effects (Barraco *et al.*, 1983; Phillis *et al.*, 1986).

Rosen and Berman (1987) first reported that focal injection of the

adenosine analog (L)-phenylisopropyladenosine inhibited kindled seizures in the amygdala, hippocampus and caudate nucleus. We have demonstrated that focal injection of 2-chloroadenosine into the rat prepiriform cortex potently suppressed convulsions elicited by a unilateral injection of bicuculline methiodide in this brain area (Franklin *et al.*, 1988). In addition to inhibition of BMI-induced convulsions, we have shown that pmol doses of the adenosine analog, N-ethylcarboxamidoadenosine, effectively prevents kainic acid-induced seizures elicited from the PPC (Zhang *et al.*, 1990). Pharmacological characterization of the adenosine receptor mediated anticonvulsant effect in this brain region, revealed a significant correlation between the potency of adenosine analogs to suppress BMI-induced convulsions *in vivo* and their respective affinities for A_1 adenosine receptors *in vitro* (Franklin *et al.*, 1989; Zhang and Murray, 1991). These data suggest that an adenosine A_1 receptor population in the prepiriform cortex is a fundamentally important element in the modulation of seizure susceptibility in this brain area.

There is growing interest in the manipulation of adenosine levels as a potential therapeutic approach in many cardiovascular (De Jong *et al.*, 1991; Schrader, 1991; Oei *et al.*, 1991) and central nervous system disorders (Phillis and O'Regan, 1989; Lin and Phillis, 1992; Marangos *et al.*, 1990; Marangos and Miller, 1991). Several pharmacological tools are available to influence the synaptic availability of adenosine. Among these are the adenosine kinase inhibitors, 5´-Amino-5´-deoxyadenosine (5´$NH_2$5´dADO) and 5´-iodotubercidin

(Miller *et al.*, 1979; Davies *et al.*, 1984 and 1986; Newby *et al.*, 1987); the adenosine deaminase inhibitors, 2´-deoxycoformycin (2´DCF) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Skolnick *et al.*, 1978; Agarwal, 1982; Padua *et al.*, 1990); the ecto-5´-nucleotidase inhibitor, α,β-methylene adenosine diphosphate (AOPCP) (Collinson et al., 1987); and the nucleoside transport blockers, nitrobenzylthioinosine (NBPMR) and dilazep (Hammond and Clanachan, 1984; Geiger *et al.,* 1988; Deckert *et al.*, 1988). Although both adenosine transport blockers and adenosine deaminase inhibitors have been used to alter purinergic modulation both *in vitro* and *in vitro* (Hertz, 1991; Geiger *et al.*, 1991), the functional effects of adenosine kinase inhibitors and of ecto-5´-nucleotidase inhibitors have received less attention. The objective of the present study was to assess the anticonvulsant efficacy of exogenous and endogenous adenosine in the PPC. The results of these studies suggest that endogenous adenosine exerts a tonic inhibitory neuromodulation in the PPC.

Materials and Methods

Animals and Stereotaxic surgery

Male Sprague-Dawley rats (Simonson Laboratories, Gilroy, CA), weighing 320-390 g, were used in these experiments. Animals were housed in groups of 4-6 upon delivery and maintained with food and water provided *ad libitum* for at least one week prior to use. Animals were kept at 22°C on a standard 12-hour light/dark schedule.

Under Equithesin anaesthesia (Equithesin 3.5 ml/kg, i.p.) rats were placed in a Kopf small animal stereotaxic instrument with incisor bar set to an elevation of -3.2 to -3.5 mm from the interaural line to establish a "flat brain" orientation in accordance with the atlas of Paxinos and Watson (1982). Animals were then unilaterally implanted with 22-gauge stainless-steel guide cannulas (Plastic Products, Roanoke, VA) such that the tips of their corresponding 28 gauge injection cannulas, which always extended from the guide lumen a minimum of 1.0 mm, were directed to a location within the right prepiriform cortex (PPC) lying 1.8 - 2.1 mm anterior and 3.3 mm lateral to bregma at a depth of 6.5 mm below dura. After implantation, the guide cannulas were fixed to the skull and to one stainless-steel anchoring screw threaded into adjacent bone, by means of dental acrylic (Kerr manufacturing Co., Romulas, MI). Patency of the injection path to the PPC was maintained by the presence of a 28-gauge stainless-steel stylet removed only to allow for introduction of injection cannulas at the time of experiment.

Drugs and Microinjections

(-)-Bicuculline methiodide (BMI), 8-(*p*-sulfophenyl)theophylline(8-*p*SPT) and 5′-iodotubercidin were purchased from Research Biochemical Inc. (Wayland, MA, U.S.A.). Nitrobenzylthioinosine 5′-monophosphate (NBPMR-PO_4) and dilazep were generous gifts of Drs. A. J. R. Paterson* and A. S. Clanachan** (*McEachern Laboratory and **Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada). Kainic acid (KA), 5′-amino-5′-deoxyadenosine p-toluenesulanate salt (5′$NH_2$5′dADO), nitrobenzylthioinosine (NBPMR, 6-(4-nitrobenzylmercapto)purine ribonucleoside), α,β-methylene adenosine diphosphate sodium salt (AOPCP), papaverine hydrochloride, adenosine hemisulfate salt and 5-amino-4-imidazolecarboxamide riboside (AICAr) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). 2′-Deoxycoformycin (2′DCF) were generously supplied by Dr. D. Herzig (Parke Davis/Warner Lambert Co., Ann Arbor, MI, U.S.A.). All drugs were prepared in a saline vehicle with final solution pH ranging from 5 to 7.

Intracranial microinjections were performed as described previously (Franklin *et al.*, 1989). The 28 gauge injection cannula was connected with a PE 20 tubing to Hamilton microsyringe (1 μl) mounted in a Harvard infusion pump with operation controlled with an electronic timer. Animals were unrestrained during injections and all drugs tested were unilaterally delivered to the specific site in the prepiriform cortex via the injection cannula at a rate of 120 nl/min.

Injection volume was 500 nl with the exception of BMI and KA (120 nl). The injection cannula was left in place for 1 min after delivery of injections to allow for complete diffusion of compounds from the cannula tip.

Behavioral paradigm

Following drug injection, animals were transferred into individual plexiglass testing chambers for observation of seizure activity. The observation period for motor seizure behavior induced by BMI and KA was 0.5 and 2 hours subsequent to infusion of the chemoconvulsant, respectively. During the observation period, a continuous behavioral record was compiled and the highest seizure score for each animal was recorded.

Behavioral seizures induced by either BMI or KA were quantified by a seizure scoring system derived from that of Racine (1972) with an ascending order of seizure severity: 0 - no seizure; 1 - myoclonic jerks of the contralateral forelimb; 2 - mild forelimb clonus (with/without mouth and facial movements - clonus of jaw and vibrissae and head nodding) lasting at least 5 sec; 3 - severe forelimb clonus lasting more than 15 sec; 4 - rearing in addition to forelimb clonus; 5 - loss of balance and/or falling in addition to rearing and forelimb clonus.

Experimental protocol

Animals were allowed a period of 24 - 48 hour to recover from surgery prior to experimentation. All experiments were carried out during the 12 hour light cycle.

Verification of cannula placement in the prepiriform cortex is inferred from the behavioral response of animals to microinjection of 118 pmol of BMI. Cannula implantation within a pharmacologically sensitive area of the rat prepiriform cortex is functionally defined by an initial positive response to the challenge dose of BMI (seizure score $\geq$ 4). Those rats with seizure scores of 4 and 5 on day 1 were used in subsequent experiments. On the day 2 adenosine and adenosine potentiators were pre-administered (either 15 or 25 min) prior to BMI challenge. The adenosine receptor antagonist 8-*p*SPT was co-injected with a chemoconvulsant. A response to drug treatment on day 2 was accepted only if a seizure response equal to control response to BMI (118 pmol) was obtained on day 3. Animals displaying a reduced seizure score on post-test day 3 as compared to the initial response to BMI on day 1, were excluded from data analysis.

Cannula placement was examined histologically in all animals showing a loss in sensitivity to BMI, and in those failing to show initial sensitivity to the convulsant. The majority of animals showed sustained sensitivity to BMI and cannula placement was sampled histologically in this population at the end of their participation in experiments.

Data analysis

Rankings of behavioral seizures were analyzed by a one-tailed Rank Sum test (Devore and Peck, 1986) to determine significant differences among mean seizure score data. Protective responses from administration of compounds prior

to BMI (118) injection were expressed as percent protection, which was calculated as percent reduction of mean control seizure score.

Dose-response data were analyzed by iterative fitting of a four parameter logistic equation through the use of the public procedure FITFUN on the PROPHET computer system.

The equation used was :

$$E = \frac{E_{max}}{1 + (K/[X])^n}$$

Where E = response; E_{max} = maximal response; K = ED_{50}; X = dose; n = a slope factor. Estimates for ED_{50} and E_{max} values are reported as means $\pm$ S.D.. Statistical difference in two sample comparison of ED_{50} values was assessed by means of the two-tailed Student's t-test.

Results

In an effort to assess the anticonvulsant efficacy of the nucleoside neuromodulator adenosine, the dose-response effects of focally administered exogenous adenosine in the PPC against BMI-induced seizures were evaluated. Tab.IV-1 shows the mean seizure scores of animals (n=4-6) after each of two treatments: an initial control injection of BMI (118 pmol) alone on day 1, and a second experimental injection of BMI administered 15 min following adenosine pretreatment on day 2. Control experiments showed that the behavioral response of animals pretreated with saline vehicle 15 min prior to BMI was indistinguishable from that of animals treated with BMI alone. As shown in Table IV-1, adenosine treatment protected rats from BMI-induced seizures in a dose-dependent manner with an ED_{50} value $\pm$ S.D. of 48.1 $\pm$ 8.4 nmol/rat. Furthermore, the protective effect of adenosine at a dose of 95 nmol was significantly attenuated by simultaneous administration of the adenosine receptor antagonist 8-*p*SPT (Tab.IV-1).

In order to define the metabolic pathways critical for the elimination of extracellular adenosine following focal administration in the PPC, the influence of inhibitors of adenosine kinase, adenosine transport and adenosine deaminase on the anticonvulsant effects of exogenous adenosine were determined. Figure IV-1 depicts the dose-response curves for adenosine against BMI seizures in the absence (control) and in the presence of either an adenosine kinase inhibitor, 5´$NH_2$5´dADO; the adenosine transport blockers, dilazep and NBPMR-PO_4; or

the adenosine deaminase inhibitor, 2´DCF. Doses of 5´$NH_2$5´dADO (0.2 nmol), dilazep (0.5 nmol), NBPMR-PO_4 (17.8 nmol) and 2´DCF (11.2 nmol), which alone were negligibly effective (5-6% protection) in altering the seizure severity alone, markedly potentiated the anticonvulsant action of adenosine. The ED_{50} values of adenosine derived from logistic fits to the dose-response data, in the context of the treatments described above, are summarized in Tab.IV-2. As shown in Tab.IV-2, the anticonvulsant potency of adenosine was significantly enhanced by all treatments, with 5´$NH_2$5´dADO producing the most profound effect.

The role of endogenous adenosine in the regulation of seizure susceptibility was next addressed by focal injection of inhibitors of either adenosine metabolism or transport alone prior to BMI-challenge. The use of potent and specific nucleoside transport inhibitors, NBPMR, was of limited utility in these studies due to its low aqueous solubility. In preliminary studies, the highest dose of NBPMR injected (1.2 nmol) in the prepiriform cortex had a marginal effect on BMI-induced seizures (14% protection). The dose-response relationships for the more water soluble adenosine transport inhibitors, dilazep, NBPMR-PO_4 and papaverine, to suppress BMI-induced convulsions are depicted in Fig.IV-2. Focal injection of dilazep (0.5 - 50 nmol) provided a potent and efficacious protection against BMI-induced seizures (Fig.IV-2) with an ED_{50} value $\pm$ S.D. of 5.6 $\pm$ 1.6 nmol/rat. As illustrated in Figure IV-2, the anticonvulsant effects produced by NBPMR-PO_4 and papaverine were also dose-

dependent. Higher doses of NBPMR-PO_4 and papaverine could not be tested due to limitations in aqueous solubility. NBPMR-PO_4 and papaverine, however, appeared to be about 30-fold less potent than dilazep against BMI-induced seizures.

The anticonvulsant effects of inhibitors of adenosine kinase and adenosine deaminase are depicted in Figure IV-3. Pretreatment with the adenosine kinase inhibitor 5´$NH_2$5´dADO afforded protection from BMI-induced convulsions in a potent and highly efficacious manner; the ED_{50} value was 2.6 $\pm$ 0.8 nmol with maximally effective doses affording complete protection against seizures. As shown in Figure IV-3, another adenosine kinase inhibitor, 5´-iodotubercidin, also efficaciously and potently inhibited BMI-seizures in a dose-dependent fashion with an ED_{50} value of 4.0 $\pm$ 2.7 nmol. The adenosine deaminase inhibitor, 2´DCF, was also effective although approximately 5-8 times less potent (ED_{50} =19.7 $\pm$ 0.4 nmol) than the adenosine kinase inhibitors. In addition, it should be emphasized that the maximal response achieved with 2´DCF administered alone was only 30% of the maximum response obtained with 5´$NH_2$5´dADO. When a dose of 5´$NH_2$5´dADO (0.2 nmol) which was inactive alone was co-administered with 2´DCF, the efficacy of 2´DCF was significantly (Student's t-test, $p < 0.01$) augmented with 2´DCF conferring nearly complete protection from BMI-induced convulsions (Fig.IV-3). The anticonvulsant potency and maximal effect for the inhibitors of adenosine kinase and adenosine deaminase is presented in Tab.IV-3.

Additional experiments assessed the effects of the nucleoside precursor AICAr. Administration of AICAr 15 min prior to BMI challenge in the prepiriform cortex (n=4) had no effect on BMI-induced seizures at doses as high as 116 nmol (data not shown).

The proconvulsant effect of a specific adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (8-*p*SPT) was investigated in both bicuculline methiodide- and kainic acid-induced seizure models. In preliminary experiments, using doses of 10, 30 and 59 pmol BMI resulted in mean seizure scores of 1.5 (n=2), 1.69 (n=13) and 3.0 (n=7), respectively (Tab.IV-4). The dose-response relationship for convulsions induced by KA in the PPC has been investigated in our previous study (Zhang *et al.*, 1990). Therefore, doses (30 pmol BMI and 100 pmol KA) which were lower than ED_{50} values for convulsions were used in the proconvulsant paradigm. The distribution of maximum seizure scores for each treatment group are shown in Table IV-5. The 1.6 nmol dose of 8-*p*SPT administered alone to five animals, produced a single mild seizure in only one animal (Tab.IV-5). When 8-*p*SPT (1.6 nmol) was co-administered with either BMI or KA, however, 8-*p*SPT significantly increased the mean seizure scores of these chemoconvulsants in animals as compared to BMI or KA treatment alone. These results demonstrate the proconvulsant activity of a focally administered adenosine receptor antagonist.

Given the proconvulsant effects of 8-*p*SPT, we further explored the functional role of endogenous adenosine in the PPC by assessing the effects of

an ecto-5´-nucleotidase inhibitor, AOPCP. The results obtained with the ecto-5´-nucleotidase inhibitor AOPCP were of particular interest, inasmuch as this compound effected a dose-dependent production of generalized behavioral motor seizures following unilateral focal injection in the PPC. At a dose of 100 nmol AOPCP elicited the severe seizure activity (seizure score $\geq$ 4) in 80 % of animals (n=5), with an average ($\pm$S.D.) latency time of 14.5 $\pm$ 1.5 min for initial clonic episode (i.e. score $\geq$ 1) and an average ($\pm$S.D.) latency of 18.2 $\pm$ 4.4 min to reach maximum seizure stage. The behavioral characteristics of AOPCP-induced convulsions were qualitatively identical to those of bicuculline methiodide-induced seizures. The dose-response data for convulsion induced by AOPCP are illustrated in Figure IV-4. The ED_{50} value for convulsions induced by AOPCP is 37.3$\pm$22.7 nmol/rat.

Discussion

Adenosine is an ubiquitous purine nucleoside. The pathways of adenosine synthesis and catabolism are depicted in Figure IV-5. Membrane-bound 5´-nucleotidase (ecto-5´-nucleotidase) appears to be the most important source of extracellularly-formed adenosine (MacDonald and White, 1985). Intracellular adenosine derives either from 5´-AMP via the action of cytosolic 5´-nucleotidase or from S-adenosylhomocysteine (SAH) via the action of SAH hydrolase. The degradation of adenosine occurs mainly through two enzymes, adenosine kinase and adenosine deaminase, which are thought to be predominantly cytoplasmic enzymes (Schrader,1983; Deckert et al., 1988; Geiger *et al.*, 1991). The nucleoside transport system which recognizes adenosine appears to operate as a bidirectional, facilitated diffusion process (Paterson *et al.*, 1985; Wu and Phillis, 1984; Deckert et al., 1988; Hertz, 1991). Consequently, the direction of adenosine flux through the transporter is a function of the adenosine concentration gradient across the cell membrane. These metabolic and transport pathways were evaluated as sites for the potential manipulation of adenosine levels using selective pharmacological agents.

If adenosine is an endogenous antiepileptic agent in the PPC, then exogenous administration of adenosine would be predicted to exert an anticonvulsant effect. The efficacy of adenosine in protecting against BMI-induced seizures we report here supports this hypothesis. It appears unlikely that adenosine produced its anticonvulsant effect through the adenosine metabolites

inosine and hypoxanthine, since we have previously reported that metabolically-stable adenosine analogs potently suppress BMI-induced seizures in a consistent with their respective potencies at A_1 adenosine receptors (Franklin *et al.*, 1988; Zhang and Murray, 1991). The anticonvulsant effect of adenosine, like those of the metabolically stable adenosine analogs (Franklin *et al.*, 1988; Zhang *et al.*, 1990), is antagonized by the adenosine receptor antagonist 8-*p*SPT (Tab.IV-1), suggesting that this response of adenosine is subserved by activation of cell surface adenosine receptors. The potent seizure suppressant effects of adenosine and adenosine analogs in the PPC, may be related to the high density of A_1 receptors in this brain area (Goodman and Synder, 1982; Lee and Reddington, 1986; Weber *et al.*, 1990).

The anticonvulsant potency of adenosine reported herein is two to three orders of magnitude lower against BMI-induced seizures than the potencies of metabolically stable adenosine analogs tested in our previous studies (Franklin *et al.*, 1989; Zhang et al., 1990). The relative low affinity of adenosine for adenosine receptors (Schwabe and Trost, 1980) and its rapid removal of adenosine from the extrasynaptic space through cellular uptake and metabolism may account for the comparatively low potency of adenosine against BMI-induced seizures. The enhancement of the anticonvulsant potency of adenosine produced by inhibitors of adenosine kinase, deaminase and transport suggest that, in the presence of high levels of exogenous adenosine, these processes contribute significantly to the synaptic elimination of adenosine.

To address the role of adenosine metabolism and transport in regulating the tonic inhibitory influence of endogenous adenosine in the PPC, pharmacological manipulations designed to increase extracellular adenosine were explored as potential anticonvulsant strategies. In support of such a tonic inhibitory modulation we previously reported that the adenosine transport blocker, dilazep, potently and efficaciously suppresses KA-induced generalized seizures (Zhang *et al.*, 1990). The present results have confirmed and extended our report of the anticonvulsant effect of dilazep by showing that dilazep elicits a dose-dependent protection against BMI-induced seizures. In accordance with these findings, it has been shown that NBPMR reduced epileptiform activity in human epileptogenic cortical slices maintained *in vitro* (Kostopoulos *et al.*, 1989) and that intravenous administration of dipyridamole lengthened the interictal phase of recurrent generalized seizures in cats (Eldridge*et al.*, 1989). The importance of the nucleoside transport system in the regulation of extracellular adenosine level was also evaluated using adenosine transport blockers in recent studies employing microdialysis. The focal administration of dipyridamole by microdialysis has been shown to significantly enhance the basal extracellular concentration of adenosine by 2-fold in piglet frontal cortex (Park and Gidday, 1990) and in rat striatum (Ballarin *et al.*, 1991). Moreover, a dramatic increase by nearly 5 fold of basal adenosine level was observed following the administration of a combination of three transport blockers (Ballarin *et al.*, 1991). Taken together, the anticonvulsant action of

nucleoside transport blockers may be attributed to an accumulation of extracellular adenosine in the synaptic cleft, although the precise mechanisms subserving the elevation of interstitial adenosine concentration by these inhibitors of the bidirectional transporter has not been fully elucidated (Fredholm *et al.*, 1980; White and MacDonald, 1990).

In the present study a dose-dependent anticonvulsant response against BMI-induced seizures was also produced by microinjection of two additional nucleoside transport inhibitor NBPMR-PO_4 and papaverine (Fig.IV-2). NBPMR-PO_4 is a pro-drug of NBPMR which is dephosphorylated by 5´-nucleotidase *in vivo* (Ogbunude *et al.*, 1984). NBPMR-PO_4 was found to be approximately one order of magnitude less potent than dilazep against BMI-induced seizures in the PPC. NBPMR, NBPMR-PO_4 and dilazep have, however, been shown to be equipotent as inhibitors of both [^{3}H]NBPMR binding to the nucleoside transport site in guinea-pig brain and [^{3}H]adenosine uptake in rat brain (Hammond and Clanachan, 1984; Geiger *et al.*, 1988). The basis for the lack of correlation between the affinities of dilazep and NBPMR-PO_4 for the adenosine transporter *in vitro* and their anticonvulsant potencies *in vivo* is presently unclear. It has been suggested that dilazep may have calcium channel antagonist activities (Ionini *et al.*, 1983; Nakagawa et al., 1986). The anticonvulsant activity of this compound, therefore, could derive from both its capacity to potentiate adenosine and to inhibit calcium influx.

Previous studies have shown that 5´$NH_2$5´dADO and 5´-iodotubercidin

are potent inhibitors of adenosine kinase in many tissues (Miller et al., 1979; Davies et al.,1984 and 1986), while lacking significant direct action on A_1 and A_2 adenosine receptors in rat brain (Davies et al., 1986). Fredholm and Lloyd (1990) found that inhibition of adenosine kinase by 5´-iodotubercidin elicited a large increase in adenosine release in rat hippocampal slices. Little is known, however, of the influence of adenosine kinase inhibitors on CNS function. In the present study administration of the adenosine kinase inhibitor 5´$NH_2$5´dADO provided a remarkably potent and efficacious suppression of BMI-induced seizures (Fig.IV-3). Moreover, it is apparent that the anticonvulsant action of another adenosine kinase inhibitor, 5´-iodotubercidin, is comparable to that of 5´$NH_2$5´dADO in terms of potency and efficacy. The limited aqueous solubility of 5´-iodotubercidin precluded the use of higher doses. The augmentation of endogenous adenosine actions by adenosine kinase inhibitors, and thereby the anticonvulsant effects of these compounds, is thought to derive from an elevation of intracellular adenosine resulting from the inhibition of adenosine kinase; driven by its concentration gradient, presumably via the bidirectional transporter, cellular adenosine efflux then ensues, resulting in an increase in extracellular adenosine concentration in the compartment of the cell surface adenosine receptors. In support of this possibility the inhibitory effects of 5´-iodotubercidin in the cardiovascular system have been reported to be blocked by the adenosine receptor antagonist theophylline (Davies et al., 1986), further suggesting that the effects of adenosine kinase inhibitors are mediated through a

subsequent activation of adenosine receptors by endogenous adenosine. It should be noted that one group (Davies et al., 1984; Davies and Hambley 1986) has reported that adenosine kinase inhibitors, such as 5´-iodotubercidin also inhibit the uptake of [^{3}H]adenosine into both guinea-pig and rat brain slices. It appears likely that the observed inhibition of adenosine uptake by adenosine kinase inhibitors is a consequence of elevated levels of endogenous adenosine (Davies et al., 1986), since Wu et al. (1984) have shown that 5´-iodotubercidin is essentially inactive in inhibiting the initial rapid uptake of [^{3}H]adenosine by rat brain synaptosomes.

In contrast to adenosine kinase inhibitors, the adenosine deaminase inhibitor 2´DCF had low efficacy when injected alone (Fig. IV-3), showing a maximal response of only 30% protection against BMI-induced seizures. These data suggest that inhibition of adenosine deaminase alone is insufficient to allow endogenous adenosine to accumulate to levels necessary for maximum protection. The limited efficacy of 2´DCF is not explained by the existence of a diffusion barrier, inasmuch as this compound can penetrate cell membranes (Agarwal, 1982) and is thus capable of inhibiting the intracellular adenosine deaminase activity (Geiger *et al.*, 1991; Ballarin *et al.*, 1991). In most tissues, the K_m values of adenosine deaminase for adenosine are 1-2 orders of magnitude higher than those of adenosine kinase. This is particularly true for rat brain where an approximate 20-fold difference exists between the K_m values of the kinase and the deaminase; the maximal activities (V_{max}) of these two enzymes,

however, are similar (Arch and Newsholme, 1978). Adenosine kinase, under physiological conditions, appears to operate near saturation whereas the velocity of adenosine deaminase, operating well below saturation, is significantly lower; both processes, phosphorylation and deamination, apparently combine to degrade adenosine, but phosphorylation of adenosine by the kinase appears to predominate under normal physiological conditions (Arch and Newsholme, 1978; Schrader, 1983). Further support for this relationship comes from studies evaluating the extent of incorporation of [^{14}C]labelled adenosine into its metabolites; these studies indicate that the majority of radioactivity (77-87%) is incorporated into nucleotides in the brain of intact animals, brain slices or synaptosomes (Wu and Phillis, 1984). Such incorporation does not occur to a significant extent (3-19 % of radioactivity) into inosine and hypoxanthine underscoring the prevalence of the phosphorylation pathway over that of deamination.

The results presented above are in accord with studies based on measurement of adenosine levels in brain following inactivation of adenosine deaminase. Helland *et al.* (1983) showed little alteration in adenosine content of mouse brain following treatment with 2´DCF. This agrees well with data obtained by microdialysis or cortical cup techniques, which detect only very modest elevation of adenosine levels in rat striatum and cerebral cortex following treatment of animals with either of the adenosine deaminase inhibitors, EHNA or 2´DCF (Zetterstrom *et al.*, 1982; Ballarin *et al.*, 1991; Phillis *et al.*, 1988).

In contrast to normal physiological conditions, the levels of adenosine, inosine and hypoxanthine are dramatically increased during and following a period of ischemia or hypoxia (Zetterstrom *et al.*, 1982; Van Wylen *et al.*, 1986; Hagberg *et al.*, 1987; Phillis *et al.*, 1988), and the administration of either EHNA or 2´DCF results in an amplification of this increase of extracellular adenosine concomitant with a decrease in the levels of inosine and hypoxanthine (Zetterstrom *et al.*, 1982; Phillis *et al.*, 1988). Under these pathophysiological conditions, adenosine deamination appears to contribute significantly to adenosine degradation. Thus the pharmacological inhibition of adenosine deaminase activity may produce neuroprotective effects in models of ischemia by reducing the degradation of endogenous adenosine (Phillis and O'Regan, 1989; Lin and Phillis, 1992). This possibility is also consistent with the 2´DCF-mediated potentiation of exogenous adenosine reported herein; the addition of a relatively large amount exogenous adenosine presumably increased the degree of saturation of adenosine deaminase. Thus it may be inferred that the contribution of adenosine deamination to adenosine metabolism is dependent on the microenvironment concentration of adenosine in different pathophysiological situations.

We have shown that the maximal efficacy of the adenosine deaminase inhibitor, 2´DCF, was elevated from a ceiling response of 30% protection against BMI-induced seizures to nearly full efficacy by co-administration of a dose of the adenosine kinase inhibitor, 5´$NH_2$5´dADO, which by itself did not

affect seizure activity. In the presence of the kinase inhibitor, adenosine metabolism is apparently shunted through the deamination pathway; thus under these conditions, inhibition of adenosine deaminase by 2´DCF will likely augment the accumulation of adenosine as compared to the effect of the adenosine deaminase inhibitor administered alone.

AICAr, which has been described as a purine precursor, has been shown to increase adenosine release in rat heart under hypoxic conditions (Oei *et al.*, 1991). Moreover AICAr has been reported to inhibit homocysteine-induced seizures in spite of its virtual inactivity against pentylenetetrazol-induced seizures in mice (Marangos et al., 1990). We find that focal injections of AICAr failed to produce an anticonvulsant response against BMI-induced seizures in the PPC. The basis for this lack of the anticonvulsant activity of AICAr is not readily apparent, but resembles its inactivity against pentylenetetrazol-induced seizures (vide supra).

An alternative strategy to assess the functional role of endogenous adenosine is either to antagonize adenosine receptors or to decrease extracellular adenosine production. A subconvulsant dose of the adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline (8-*p*SPT) significantly potentiated the behavioral seizure activity induced by either BMI or kainic acid (Tab.IV-5), indicating that 8-*p*SPT exerts a proconvulsant action. 8-*p*SPT is a polar 8-phenylxanthine derivative which appears to penetrate cell membranes only to a very limited extent (Daly *et al.*, 1981 and 1982). In contrast to the

permeant methylxanthines caffeine and theophylline (Amer and Kreighbaum, 1975; Rall, 1982) the use of this impermeant and selective adenosine receptor antagonist precludes the possibility of inhibition of cytosolic phosphodiesterases in the expression of this proconvulsant response. In agreement with our results, a proconvulsant response is also observed following administration of the selective adenosine receptor antagonist cyclopentyltheophylline in a model of recurrent generalized seizures (Eldridge *et al.*, 1989). Moreover, a self-sustained epileptiform activity of hippocampal CA3 neurons produced by the A_1 selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine has been reported (Alzheimer *et al.*, 1989). These findings provide strong evidence that endogenous adenosine exerts a basal inhibitory tone which modulates seizure susceptibility in the CNS.

Ecto-5´-nucleotidase is the plasma membrane-bound enzyme, primarily responsible for production of extracellular adenosine from 5´-AMP, as illustrated in Figure IV-5. AOPCP has been shown to be a selective inhibitor of ecto-5´-nucleotidase, but not the cytosolic 5´-nucleotidases (Collinson et al., 1987). The potent convulsant activity of the ecto-5´-nucleotidase inhibitor AOPCP is an intriguing finding, which implies that this enzyme is an important source of tonically generated extracellular adenosine. The data of MacDonald and White (1985) suggest that the majority of basal extrasynaptic adenosine appears to be generated from the extracellular degradation of a released nucleotide, insofar as inhibition of ecto-5´-nucleotidase by 90 % with AOPCP

diminished basal extrasynaptosomal adenosine levels by 74 % in rat brian. Our *in vivo* data are consistent with this observation, and suggest that ecto-5´-nucleotidase activity may represent a key control point in the regulation of extracellular adenosine levels. The effectiveness of AOPCP as a convulsant in the PPC, suggests that adenosine plays a critical tonic inhibitory role in regulating the level of neuronal activity in the PPC. As such, ecto-5´-nucleotidase may represent an important etiological locus in the pathology of epileptogenesis.

Considered together these data strongly support the hypothesis that adenosine is an important endogenous anticonvulsant substance in the prepiriform cortex. Our results suggest that the seizure suppressant action of inhibitors of adenosine kinase, adenosine transport and adenosine deaminase is an indirect result of the increased occupancy, and therefore activation, of adenosine A_1 receptors which derives from an augmentation by these compounds of the synaptic availability of adenosine. These studies further document the critical role played by adenosine receptor activation in limiting the progression of pathologically excitatory neuronal activity in the PPC, and that compounds which potentiate extracellular adenosine such as adenosine kinase inhibitors, adenosine deaminase inhibitors and inhibitors of adenosine transport may represent a novel class of antiepileptic drug candidates.

Acknowledgement

We thank to Dr. D. Herzig for generously supplying us with 2'-deoxycoformycin. We also gratefully acknowledge to Drs. A.J.R. Paterson and A.S. Clanachan for the gifts of nitrobenzylthioinosine 5'-monophosphate and dilazep. This work was supported by U.S. Public Health Service Grant NS-23227 to T.F.M.

TABLE IV-1

Modulation of BMI-induced epileptic seizures in rat prepiriform cortex by focal injection of adenosine

Treatment Adenosine Dose	Distribution of Seizure Scores 0	1	2	3	4	5	Mean Seizure Score (n)	Percent Protection
Control						4	5.00 (4)	
9.5 nmol						4	5.00	0
Control					1	3	4.75 (4)	
31.6 nmol	1				2	1	3.25	32
Control					2	3	4.60 (5)	
95 nmol	3			1	1		1.40	70
Control						4	5.00 (4)	
316 nmol	3		1				0.50	90
Control						6	5.00 (6)	
95 nmol + 8-*p*SPT	1				2	3	3.83	23

All agents were unilaterally microinjected into PPC as described in methods. Either adenosine or vehicle (control) were microinjected 15 min prior to BMI (118 pmol) challenge in PPC. 8-*p*SPT (1.6 nmol) was co-administered with adenosine (95 nmol) 15 min before a challenge dose of BMI. Protective response of adenosine was expressed as percent protection: the percentage of reduction in mean seizure score of animals pre-treated with the indicated doses of adenosine compared to control (BMI 118 pmol alone).

TABLE IV-2

Influence of inhibitors of adenosine kinase, adenosine transport and adenosine deaminase on the potency of adenosine against BMI-induced seizures in rat prepiriform cortex

Treatment	Adenosine $ED_{50} \pm$ S.D.[a] (nmol)
Control	48.1 ± 8.4
+ 5´$NH_2$5´dADO (0.2 nmol)	8.8 ± 1.5***
+ Dilazep (0.5 nmol)	10.7 ± 0.4***
+ NBPMR-PO_4 (17.8 nmol)	19.9 ± 13.6*
+ 2´DCF (11.2 nmol)	16.4 ± 0.4**

[a] Determined by iterative, least squares non-linear regression analysis as described in methods. Significance of differences from control (Student's t-test) are indicated as follows: * $P<.05$; ** $P<.01$; *** $P<.001$. Other methods were described in the legend of Fig.IV-1.

TABLE IV-3

Potency and maximal effect of inhibitors of adenosine kinase and adenosine deaminase as anticonvulsants against BMI seizures in rat PPC

Treatment	ED_{50} ± S.D.[a] (nmol)	Emax ± S.D.[a] (%)
5′$NH_2$5′dADO	2.6 ± 0.8	100 ± 9.9
5′Iodotubercidin	4.0 ± 2.7	100 ± 29
2′DCF	19.7 ± 0.4	30.2 ± 0.3
2′DCF + 5′$NH_2$5′dADO (0.2 nmol)	21.2 ± 12.6	100 ± 20

[a]Determined by iterative, non-linear regression analysis as described in methods.

TABLE IV-4

Convulsant effects of bicuculline methiodide following focal injection in the rat prepiriform cortex

BMI (pmol)	Distribution of Seizure Scores 0	1	2	3	4	5	Mean Seizure Score (n)
10	0			1			1.50 (2)
30	6		3	1		3	1.69 (13)
59	2		1		1	3	3.00 (7)
118					7	8	4.53 (15)

Bicuculline methiodide was microinjected at the indicated doses unilaterally into the prepiriform cortex as described in the methods. Animals were then observed for a 30 min epoch and the mean highest seizure score attained for each group of animals of size (n) for each treatment was determined.

TABLE IV-5

Proconvulsant effects of 8-*p*-(sulfophenyl)theophylline (8-*p*SPT) on BMI- and KA-induced seizures in rat prepiriform cortex

Treatment	Dose (pmol)	Distribution of Seizure Scores						Mean Seizure Score (n)
		0	1	2	3	4	5	
8-*p*SPT	1600	4	1					0.20 (5)
BMI	30	6	1	3			3	1.69 (13)
BMI +8-*p*SPT	30 1600				1	3	1	4.00* (5)
KA	100	3				1	1	1.80 (5)
KA +8-*p*SPT	100 1600			1			3	4.25* (4)

Animals were observed for a 30 min epoch after BMI administration and for a 120 min period after KA treatment. BMI or KA were co-injected with 8-*p*SPT in a volume of 120 nl. * Mean seizure score was significantly greater ($p < 0.05$, one tailed rank-sum test) than that of BMI or KA treatment alone.

FIGURE IV-1 Potentiation of the protective action of adenosine against BMI-induced seizures in rat PPC by 5′$NH_2$5′dADO, dilazep, NBPMR-PO_4 and 2′DCF. Control represents pretreatment (15 min) of adenosine alone at the indicated doses before a BMI(118 pmol) challenge; dilazep (0.5 nmol) was co-injected with various doses of adenosine 15 min prior to BMI (118 pmol); 5′$NH_2$5′dADO (0.2 nmol), NBPMR-PO_4 (17.8 nmol), and 2′DCF (11.2 nmol) were injected 10 min before pretreatment with the indicated doses of adenosine, which was always injected 15 min prior to BMI (118 pmol) challenge. The dose-response curves were derived by fitting a logistic equation to the data using the PROPHET Public procedure FITFUN (see methods).

FIGURE IV-1

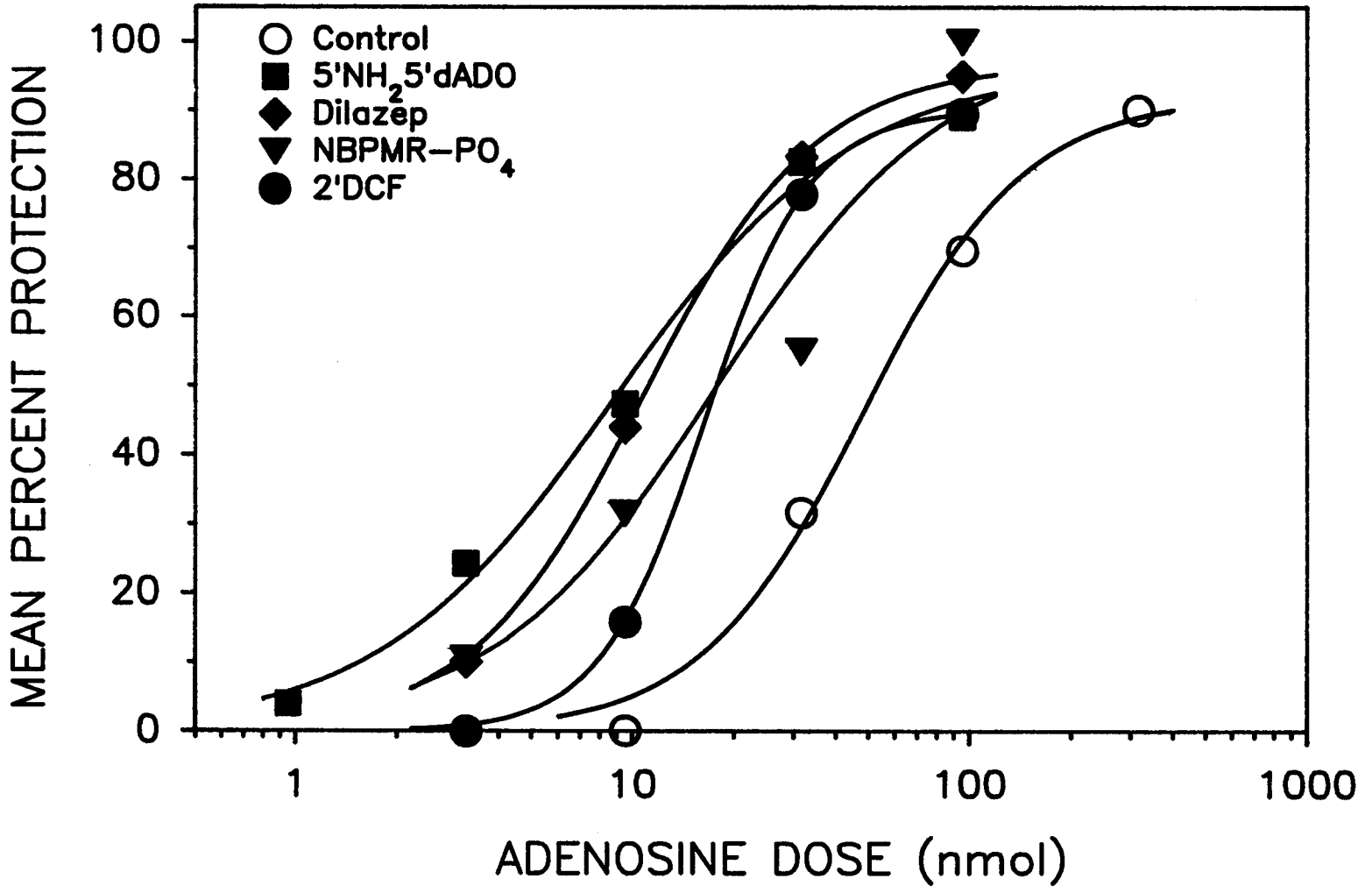

Control
5'NH2 5'dADO
Dilazep
NBPMR–PO4
2'DCF
MEAN PERCENT PROTECTION
100
80
60
40
20
0
1
10
100
1000
ADENOSINE DOSE (nmol)

FIGURE IV-2 Anticonvulsant effects of the adenosine transport blocker dilazep, NBPMR-PO_4 and papaverine against BMI-seizures. Ordinate values describe the percent reduction, from control response to BMI (118 pmol) alone, in mean seizure scores for groups (n=4-6) of animals receiving the indicated drug treatments prior to BMI (118 pmol) challenge. Dilazep and papaverine were administered 15 min prior to BMI challenge, and NBPMR-PO_4 was administered 25 min prior to BMI challenge. The symbols represent the data points of groups of animals at each dose level; the curves were obtained from expansion of the logistic equation as defined by the parameter estimates derived from non-linear regression analysis as described in methods.

FIGURE IV-2

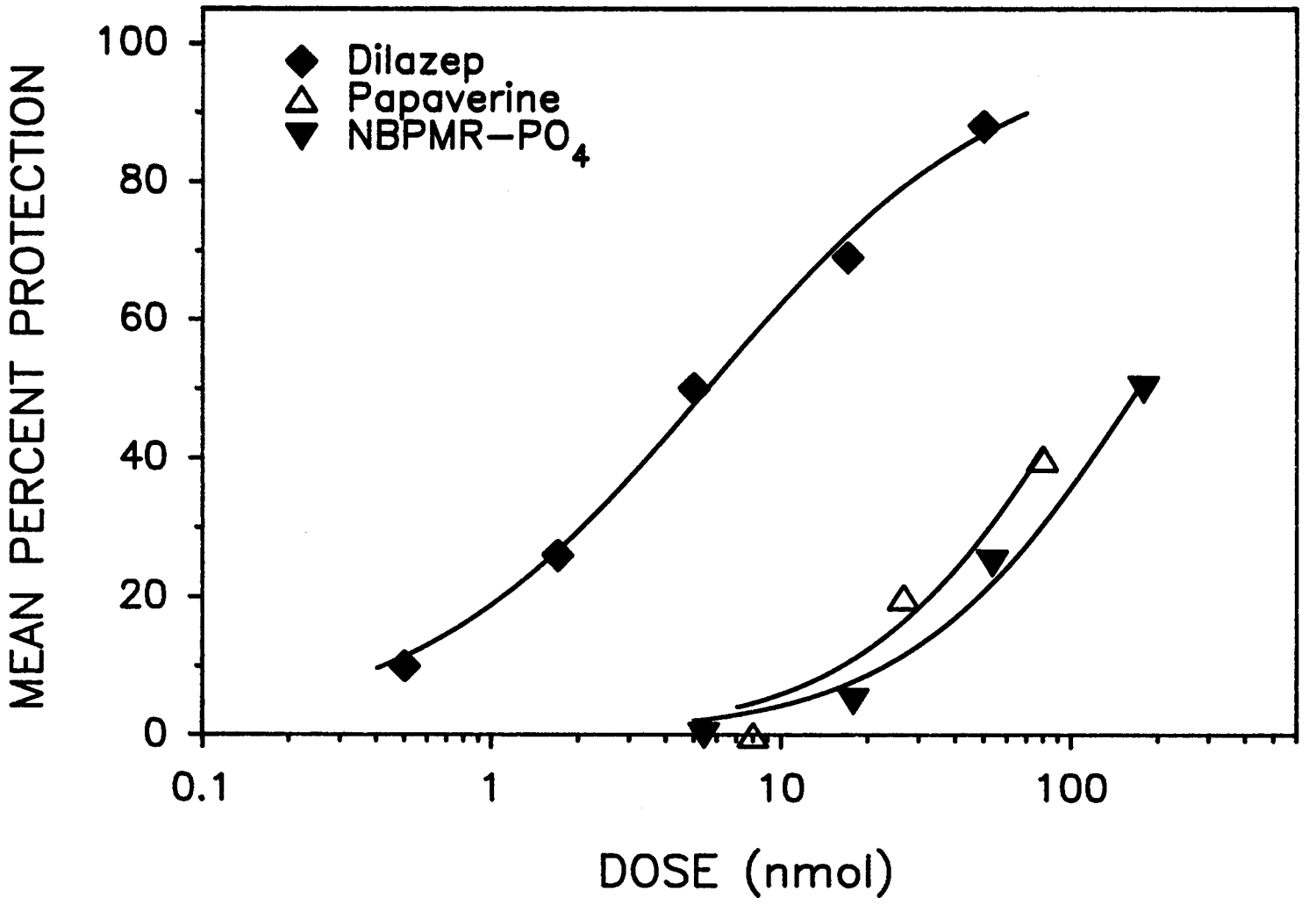

MEAN PERCENT PROTECTION
100
80
60
40
20
0
Dilazep
Papaverine
NBPMR–PO4
0.1
1
10
100
DOSE (nmol)

FIGURE IV-3 Anticonvulsant effects of the adenosine kinase inhibitors 5′$NH_2$5′dADO and 5′iodotubercidin, and the adenosine deaminase inhibitor 2′DCF on BMI-induced seizures in rat prepiriform cortex. 5′$NH_2$5′dADO, 5′iodotubercidin, 2′DCF or the combination of 2′DCF and 5′$NH_2$5′dADO (0.2 nmol) were injected, at the indicated doses, 25 min prior to the BMI challenge dose (118 pmol) in prepiriform cortex. For additional details see the legend of Fig.IV-2.

FIGURE IV-3

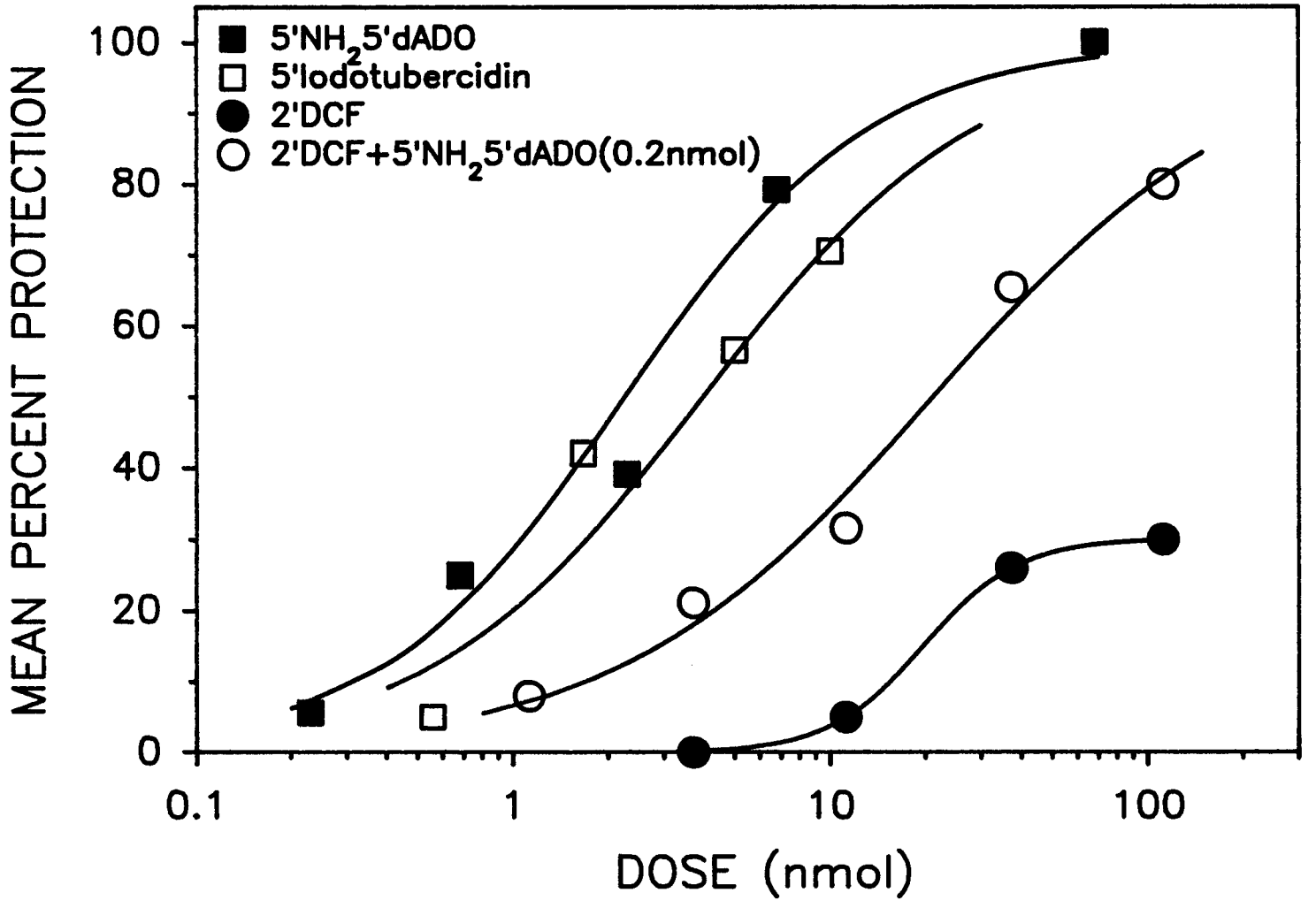

MEAN PERCENT PROTECTION
100
80
60
40
20
0
0.1
1
10
100
DOSE (nmol)
5'NH2 5'dADO
5'Iodotubercidin
2'DCF
2'DCF+5'NH2 5'dADO(0.2nmol)

FIGURE IV-4 Convulsant effects of ecto-5′-nucleotidase inhibitor α, β-methylene adenosine diphosphate (AOPCP) after focal injection in prepiriform cortex. Animals were observed for 30 min following unilateral administration of AOPCP in the PPC. Each bar value represents the mean seizure score for groups of animals (n=5-9) treated with the indicated doses of AOPCP.

FIGURE IV-4

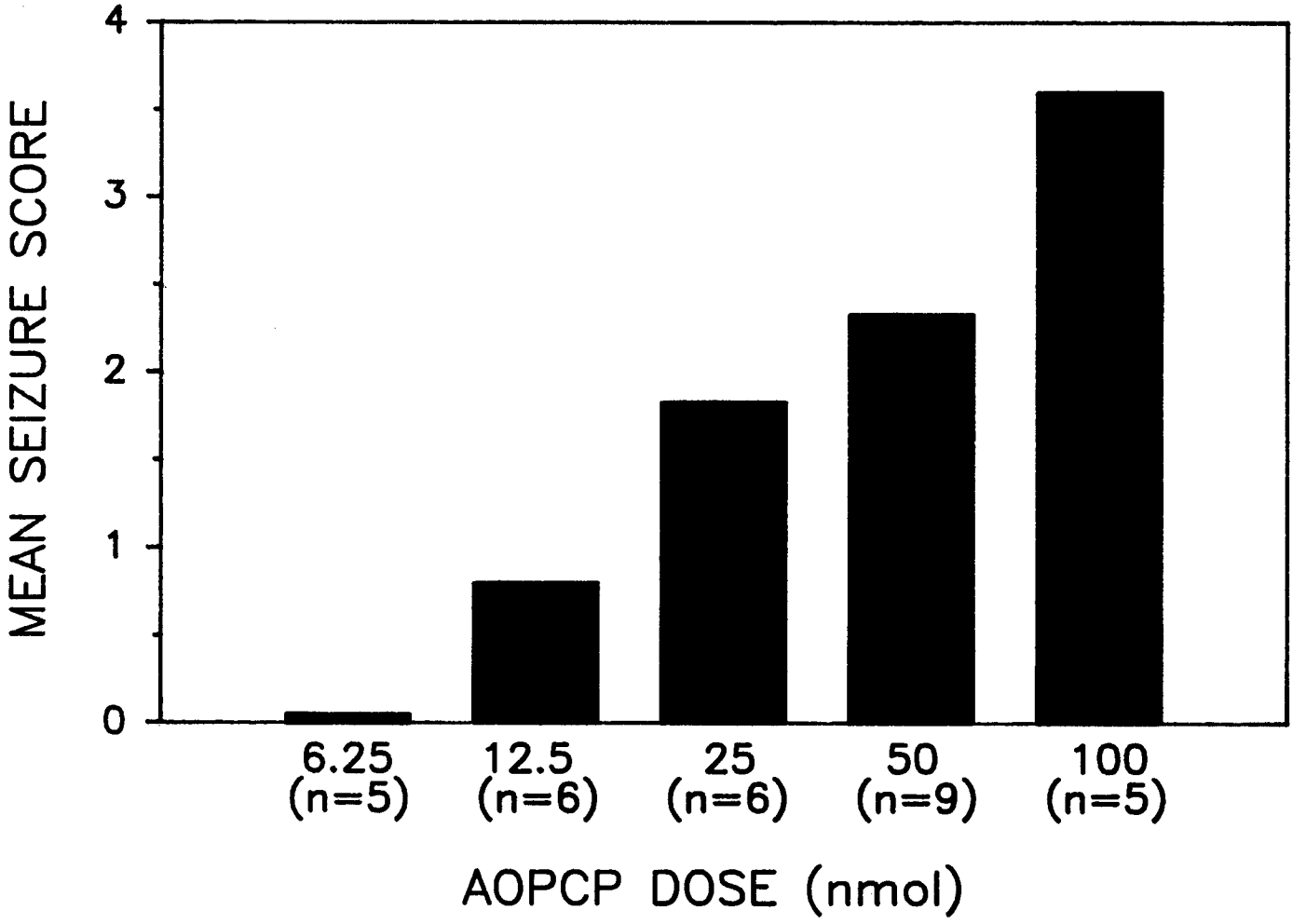
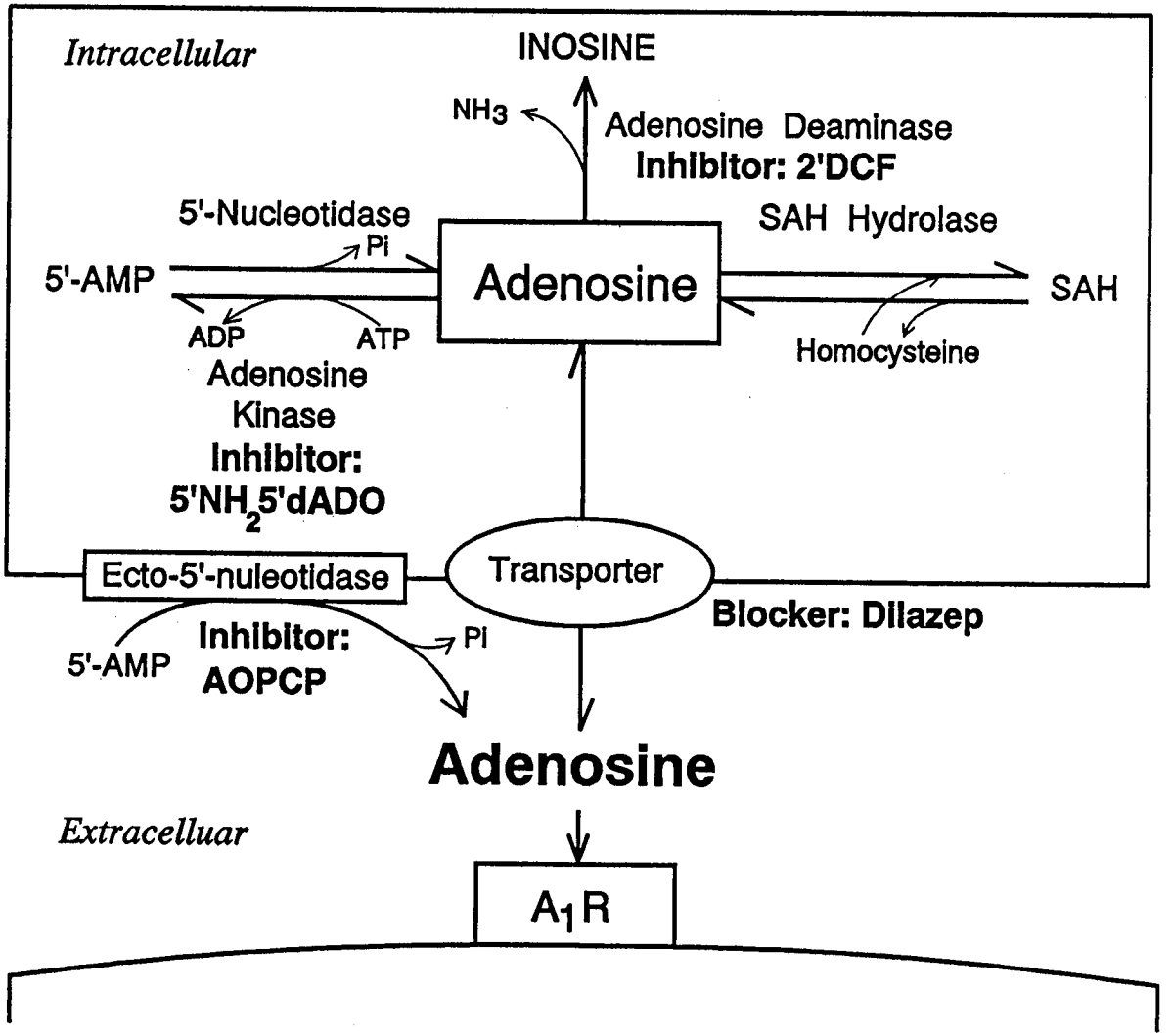

MEAN SEIZURE SCORE
4
3
2
1
0
6.25
(n=5)
12.5
(n=6)
25
(n=6)
50
(n=9)
100
(n=5)
AOPCP DOSE (nmol)

FIGURE IV-5 Targets for the manipulation of endogenous adenosine levels.

SAH: S-adenosylhomocysteine; A_1R: Adenosine A_1 receptor.

FIGURE IV-5


Intracellular
INOSINE
NH3
Adenosine Deaminase
Inhibitor: 2'DCF
5'-Nucleotidase
Pi
SAH Hydrolase
5'-AMP
Adenosine
SAH
ADP
ATP
Homocysteine
Adenosine
Kinase
Inhibitor:
5'NH2 5'dADO
Ecto-5'-nuleotidase
Transporter
Blocker: Dilazep
5'-AMP
Inhibitor:
AOPCP
PI
Adenosine
Extracelluar
A1R

CHAPTER V

ELEVATION OF BICUCULLINE SEIZURE THRESHOLD BY FOCAL ACTIVATION OF ADENOSINE RECEPTORS IN PREPIRIFORM CORTEX

Abstract

The timed infusion technique was employed to determine the seizure threshold dose of bicuculline, infused through a tail vein, required to elicit a myoclonic jerk. The adenosine analog N-ethylcarboxamidoadenosine (NECA) at dose 16 pmol was bilaterally microinjected in the rat prepiriform cortex 10 min prior to intravenous infusion of bicuculline. Pretreatment with NECA in the prepiriform cortex (PPC) significantly increased the seizure threshold to bicuculline, indicating that focal activation of adenosine receptors in the PPC protects animals from seizures initiated by systemic administration of the convulsant. Combined with earlier results in the PPC, we conclude that the A_1 adenosine receptor population in the prepiriform cortex represents a fundamentally important element in modulation of seizure susceptibility in the central nervous system.

Abbreviations: NECA, N-ethylcarboxamidoadenosine; PPC, prepiriform cortex.

Introduction

The intravenous, time-infusion technique has been demonstrated to be the most reliable method for determining seizure thresholds to a convulsant drug such as pentylenetetrazol (Nutt *et al.*, 1980 and 1981) and to be a simple screening procedure for evaluating the potential of compounds as anticonvulsants (Green and Murray, 1989). This technique has been employed in our laboratory to explore the effects of adenosine receptor agonists and antagonists on seizure thresholds to chemoconvulsants such as pentylenetetrazol, bicuculline and picrotoxin in rats (Murray *et al.*, 1985; Szot *et al.*, 1987). Previous results indicate that intravenous administration of adenosine agonists suppress seizures elicited by intravenous injection of convulsants and that the pharmacological profile is consistent with activation of A_1 adenosine receptors (Murray *et al.*, 1985). Moreover, chronic treatment of rats with the adenosine antagonist, theophylline, reveals a temporal relationship between upregulation of cerebral cortical A_1 adenosine receptors and elevation of seizure thresholds to bicuculline (Szot *et al.*, 1987; Sander and Murray, 1989). This result suggests that the cerebral cortex could be one brain region where adenosine is involved in alterations of seizure susceptibility. The relevant brain loci within the cerebral cortex at which A_1 adenosine receptors exert a modulation of seizure threshold remain to be delineated.

Observations reported in earlier chapters, strongly suggest that the prepiriform cortex, the part of primary olfactory cortex which is a

phylogenetically old type of cerebral cortex, represents an important neuroanatomical site for mediating the suppressant action of adenosine and adenosine analogs against generalized seizures evoked by unilateral focal injection of chemoconvulsants in this area. Considered together, the present study was undertaken to further characterize the adenosine receptor in the prepiriform cortex involved in modulation of seizure susceptibility for a systemically administered convulsant. The intravenous, time-infusion technique was employed to determine seizure threshold to the convulsant, bicuculline, following bilateral injection of the adenosine agonist, NECA, to stimulate adenosine receptors in the PPC. Our finding demonstrates that focal activation of adenosine receptors in the prepiriform cortex results in a reduced sensitivity to bicuculline.

Materials and Methods

Intracerebral injection in PPC

Male Sprague-Dawley rats (360-380 g), under Equithesin anaesthesia, were placed in a Kopf small animal stereotaxic instrument and bilaterally implanted with paired, 22 and 28 gauge guide and injection cannulae, respectively. With the incisor bar at -3.5 mm, the cannulae were aimed at a site in the prepiriform cortex 2.0 mm anterior and 3.3 mm lateral to bregma, at a depth of 6.5 mm below dura. Animals were allowed at least 24 hour recovery before experimentation.

In this case paired experimental design was used, and animals were randomly assigned to pretreatment of either saline or NECA (16 pmol) into the PPC 10 min prior to intravenous infusion of bicuculline. Intracerebral microinjection procedure was performed using a modification of the method as previously described (Franklin *et al.*, 1989). The 28 gauge injection cannulae were connected with PE 20 tubings to two Hamilton microsyringes (1 μl) bilaterally mounted in a Harvard syringe infusion pump (Model 22). Either saline or NECA were injected bilaterally in the PPC at rate of 0.94 nl/sec. in a volume of 120 nl.

Bicuculline seizure threshold

Seizure threshold for bicuculline (Sigma Chemical Co., St. Louis) was determined using a time-infusion method as described previously (Nutt *et al.*, 1981; Murray *et al.*, 1985; Szot *et al.*, 1987). Animals were lightly restrained and

bicuculline was infused through a 25 gauge butterfly needle inserted into the lateral tail vein. The butterfly assembly was connected with PE tubing to a syringe (5 ml) mounted in a Sage infusion pump (Sage Instruments, Cambridge, MA; Model 341). The solution of bicuculline (0.05 mg/ml) was infused at a constant rate of 1.2 ml/min. The end point of infusion was taken as the first myoclonic jerk of the head and neck, a phenomenon which has been reported to occur synchronously with the first EEG discharge (Nutt *et al.*, 1981). Seizure threshold for bicuculline is expressed in units of mg/kg of convulsant infused intravenously. The time of the first myoclonic twitch was recorded to the nearest 0.1 sec. and the dose of convulsant required to elicit the seizure was calculated from this time, the concentration of solution infused and the weight of the animal according to the formula as shown in Tab.V-1. Statistical difference of seizure thresholds between control and treated was determined by paired Student's t-test.

Histology

The cannulae placements were histologically verified at the end of the study. Animals were sacrificed by decapitation and their brains were removed rapidly and frozen over dry ice. Serial sections (16 or 32 μm) were taken coronally by IEC microtome cryostat (Damon/IEC Ltd., Bedfordshire, England) at -17°C in the vicinity of the injection cannula track. Slide-mounted sections were stained with cresyl violet and then the positions of injection cannula tips were examined under a light microscope. Animals in which the cannula placement was misdirected were excluded from data analysis.

Results

When NECA was bilaterally microinjected in the PPC at a dose of 16 pmol, there was a significant increase in the dose of bicuculline required to elicit a seizure in all animals. The seizure threshold to bicuculline for individual animals following either saline or NECA pretreatment was shown as follows:

Subject	Bicuculline Seizure Threshold (mg/kg)	
	Control	NECA
1	0.299	0.461
2	0.250	0.309
3	0.176	0.223
4	0.246	0.288
5	0.312	0.430
6	0.368	0.448

Sample distribution and mean values are illustrated in Fig.V-1. In NECA-treated condition the mean seizure threshold was increased by 19.4 %. The paired data which was analyzed by paired Student's t-test (two tailed), indicate that the seizure threshold to bicuculline after NECA pretreatment was significantly different from control ($p < 0.01$).

Bilateral cannula implantations in the prepiriform cortex were histologically examined in all animals; a representative coronal section is shown in Fig.V-2.

Discussion

Presumably, many brain regions, which not only contain a relatively high density of adenosine receptors but also are involved in seizure generalization, are likely to be important sites for the anticonvulsant action of adenosine. The work of Rosen and Berman (1987), showing microinjection of adenosine analogs at the sites of kindled foci block seizures kindled from amygdala, hippocampus and caudate nucleus, suggests that these regions may be central to the anticonvulsant effects of adenosine in kindling seizures. In accordance with this study, we have previously demonstrated that the prepiriform cortex appears to be a neuroanotomical substrate for the antiepileptic action of adenosine against focal convulsant challenge (Franklin *et al.*, 1989; Zhang *et al.*, 1990). In this study, we explored the possibility of the prepiriform cortex as an action site of adenosine against minimal seizures induced by systemic infusion of bicuculline.

NECA was selected as the adenosine receptor agonist employed in the study based on both its high potency and efficacy among adenosine analogs tested in the PPC in previous studies (Franklin *et al.*, 1989; Zhang *et al.*, 1990). The dose of NECA (16 pmol) used in the study exceeds its ED_{100} as an anticonvulsant against focal BMI challenge, implying that it may possess a high occupancy of adenosine receptors around the injection locus in the PPC.

These results indicate that bilateral injection of the adenosine analog NECA in the PPC produced an elevation of the seizure threshold to intravenous bicuculline, suggesting that focal activation of adenosine receptors protect

against minimal threshold seizures. Although the precise initiation site and spreading pathways in the CNS for epileptic seizures induced by systemic infusion of bicuculline are uncertain (Fisher, 1989), adenosine receptors in the PPC could mediate the action either by limiting epileptogenesis in the seizure origin or by preventing the propagation as a downstream component in the pathway. At this forebrain site, the GABA agonist muscimol and the glutamate receptor antagonist 2-amino-phosphonoheptanoic acid are also powerful anticonvulsants to seizures induced by i.v. bicuculline in rats (Piredda and Gale, 1986; Piredda *et al.*, 1985). These studies combined with our findings suggest that the prepiriform cortex appears to be involved in the development of bicuculline seizures.

It seems that distinct neuroanatomical pathways and/or neurochemical mechanisms may underlie different types of seizures (Fisher, 1989; Meldrum, 1990). Thus, it is possible that the effects of focal injection of adenosine may vary from one site to another in a wide range of experimental seizure models. Recent investigation (Sarro *et al.*, 1991) has shown that infusion of the adenosine analog, 2-chloroadenosine, into the substantia nigra protects rats against electroshock seizures and also protects genetically epilepsy prone rats against audiogenic seizures. Furthermore, the latter studies have indicated that audiogenic seizures were potently inhibited by focal injection of 2-chloroadenosine into the inferior colliculus which is a critical point in the auditory pathway, but that seizures were not suppressed when 2-ClA was infused

into the endopeduncolar nucleus. These data permit the authors to conclude that audiogenic seizures and electroshock seizures may be modulated by adenosine at the levels of the inferior colliculus and substantia nigra.

The results of our experiment provide additional support for the hypothesis that the adenosine receptor population in the prepiriform cortex plays a significant role in modulation of seizure susceptibility. Future studies should, therefore, attempt to investigate additional neuroanatomical sites which are important for adenosine in the alteration of seizure generation.

Acknowledgement

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TABLE V-1

Method for the determination of bicuculline seizure threshold in rat

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1. Bicuculline is continuously infused into a lateral tail vein of the rat via a 25 gauge butterfly

2. The time (sec) to the first myoclonic twitch is recorded

3. The bicuculline seizure threshold is calculated from the recorded time (min), the infusion rate of bicuculline (1.2 ml/min), the concentration of bicuculline (0.05 mg/ml) and the weight (kg) of the animal

---

Bicuculline Seizure Threshold (mg/kg)

$$= \frac{\text{time (min) x infusion rate (ml/min) x bicuculline conc. (mg/ml)}}{\text{weight (kg)}}$$

FIGURE V-1 Anticonvulsant effect of bilateral focal injection of NECA in the prepiriform cortex against intravenous bicuculline seizure threshold. Either saline (control) or NECA (16 pmol) was bilaterally administered into the PPC 10 min prior to intravenous infusion of bicuculline. Open- and filled circles are control and treated data of bicuculline seizure threshold, respectively. The horizontal lines indicate the mean seizure threshold values from saline and NECA treatments. Asterisk indicates significant difference from control (paired Student t-test, $p < 0.01$).

FIGURE V-1

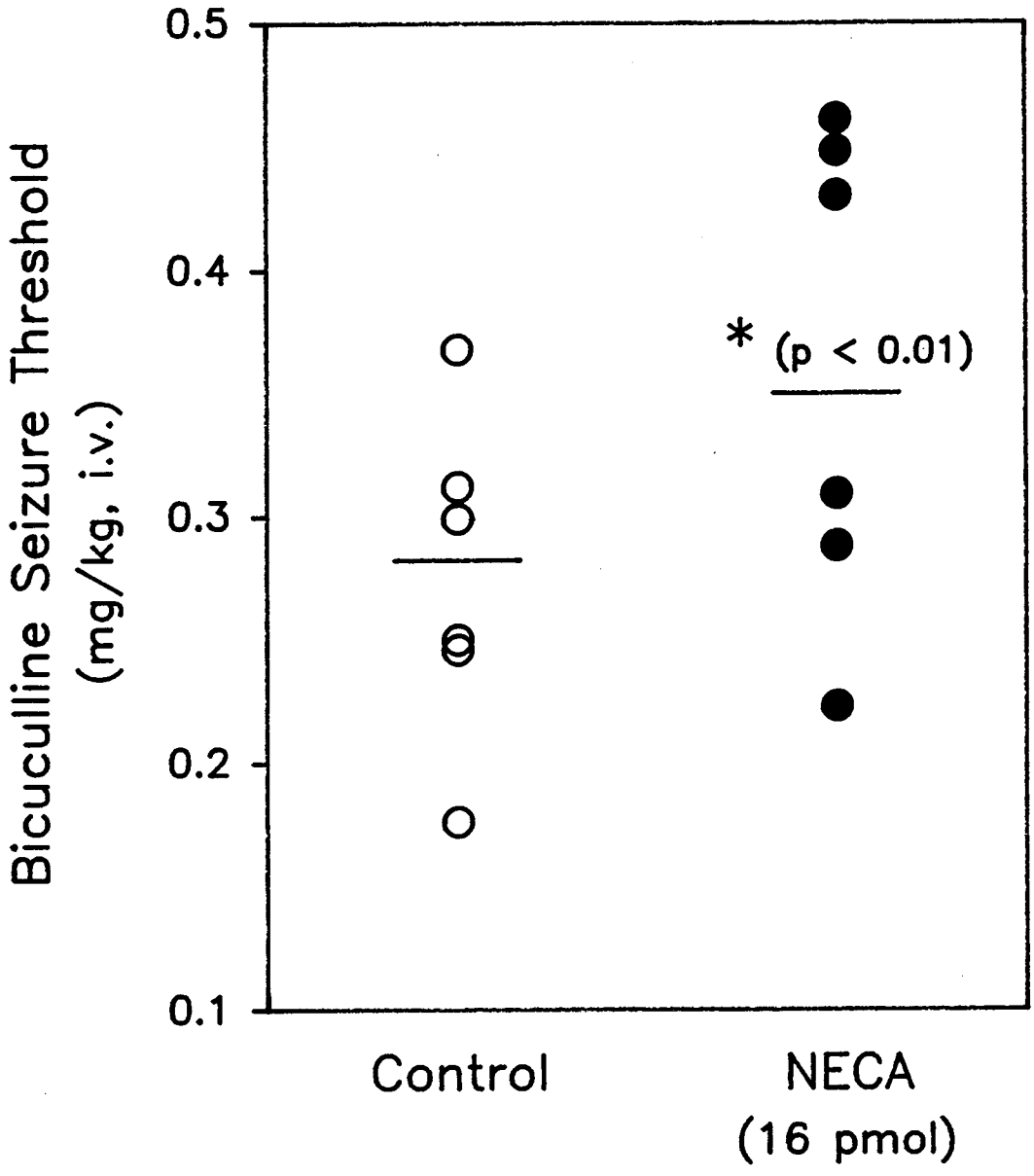
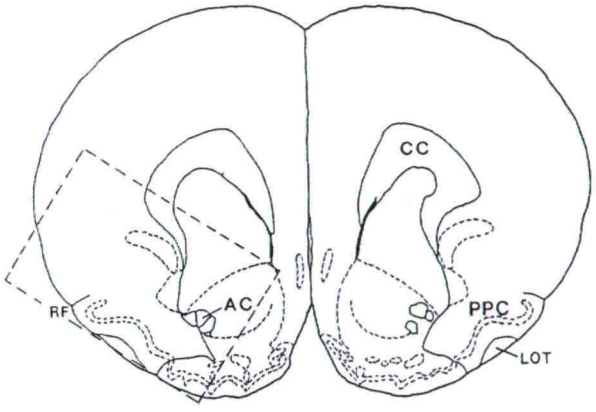
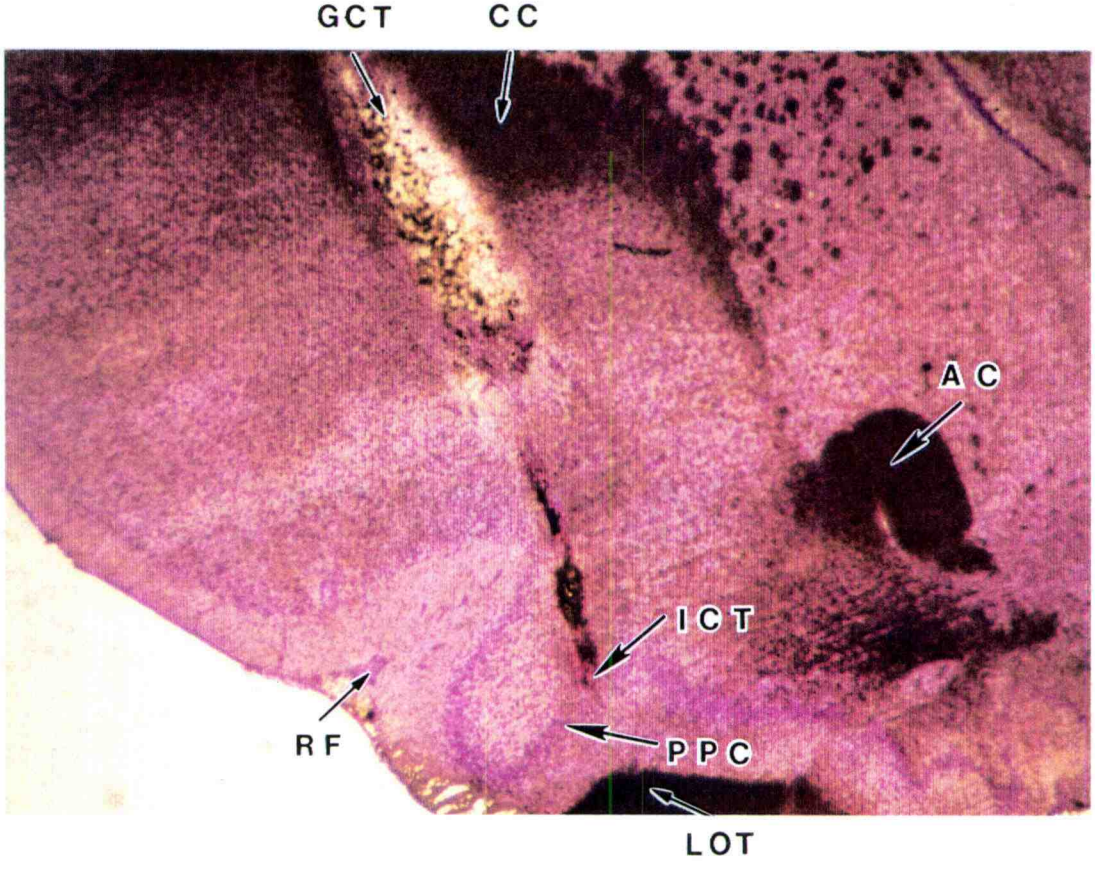

Bicuculline Seizure Threshold
(mg/kg, i.v.)
0.5
0.4
0.3
0.2
0.1
* (p < 0.01)
Control
NECA
(16 pmol)

FIGURE V-2 A representative example of the microinjection site in the rat prepiriform cortex. The color photo only shows the part of coronal section of rat brain stained by cresyl violet. Abbreviations: AC: anterior commissure; CC: corpus callosum; GCT: guide cannula tract; ICT: injection cannula tip; LOT: lateral olfactory tract; PPC: prepiriform cortex; RF: rhinal fissure.

FIGURE V-2





CHAPTER VI

SUMMARY AND CONCLUSION

In the 18 years since the initial observation by Maitre *et al.* (1974) that adenosine prevented audiogenic seizures in mice, a great deal has been discovered about the role of adenosine in epilepsy. The anticonvulsant effects mediated through adenosine receptors in a discrete forebrain region, the prepiriform cortex which may be involved in seizure generalization, have been pharmacologically characterized in the present study. The pharmacological tools included adenosine receptor agonists, an adenosine receptor antagonist, nucleoside transport blockers as well as enzymatic inhibitors interfering with either adenosine formation or degradation. The potential anticonvulsant efficacy for many compounds was evaluated using an unique epileptic seizure model in which generalized motor seizures can be initiated by unilateral focal injection of a very low dose of a chemoconvulsant in the prepiriform cortex. This intracerebral microinjection procedure was performed to deliver adenosine-related compounds into the same locus within the PPC.

The chemoconvulsant kainic acid, which is an agonist for one subtype of glutamate receptor, produces dose-dependent generalized seizures following focal injection in the PPC. The adenosine analog NECA, when co-injected with KA, protects animals from convulsions in a dose-dependent and highly potent manner. Morevover, the anticonvulsant action of NECA is completely abolished

by the adenosine antagonist 8-pSPT, suggesting that activation of adenosine receptors in this brain area underlies the anticonvulsant efficacy of the adenosine analog. As illustrated in Fig.VI-1, KA-induced seizures elicited from the PPC could result from a presynaptic process such as stimulating excitatory amino acid release. The localization of A_1 adenosine receptors in axon terminals of excitatory neurons and A_1 adenosine receptor mediated inhibition of neurotransmitter release has been well characterized in many brain regions. In this study, the ability of the adenosine agonist NECA and the nucleoside transport blocker dilazep to inhibit KA-induced convulsions may be partly due to presynaptic inhibition of excitatory neurotransmitter release in the domain of output neuron of the PPC (Fig.VI-1).

Following focal injection of adenosine analogs, CGS21680, an agonist which has both high affinity and selectivity for A_2 adenosine receptors, is the least potent anticonvulsant among adenosine analogs including nonselective- and selective A_1 and A_2 agonists tested in the PPC. Using [^{3}H]DPCPX as a selective A_1 antagonist radioligand to label A_1 receptors in frontal cerebral cortical membrane preparations, competition studies were performed to examine the affinity of these adenosine analogs for the A_1 adenosine receptor. These studies demonstrated that the potency of adenosine analogs as anticonvulsants *in vivo* is significantly correlated with their respective affinity for A_1 adenosine receptors. This functional study is also consistent with the distribution of A_1 and A_2 adenosine receptors in the corresponding brain region. It appears that the

anticonvulsant effects of CGS21680 is mediated via A_1 adenosine receptors in the prepiriform cortex.

Pharmacological manipulation of endogenous adenosine in the PPC is a strategy to affect seizure expression. The adenosine kinase inhibitors, 5′$NH_2$5′dADO and 5′iodotubercidin, and the nucleoside blocker, dilazep, are highly efficacious and potent anticonvulsants against BMI-induced seizures. The adenosine deaminase inhibitor, 2′DCF, had a much low efficacy only yielding 30% of maximal protection as compared to the kinase inhibitors. These results suggest that elevation of endogenous adenosine level may contribute to seizure suppression, and that adenosine kinase and adenosine transport system may play a pivotal role in the clearance of extracellular adenosine. The proconvulsant effects of the adenosine antagonist 8-pSPT observed in both BMI- and KA-induced epileptic seizure models may result from blockade of endogenous adenosine action at adenosine receptors. Moreover, reduction of local adenosine formation in the extracellular compartment by focal injection of an ecto-5′-nucleotidase inhibitor, AOPCP, into the PPC produced bilateral convulsions. These findings not only suggest that adenosine receptor-coupled processes may be involved in the etiology underlying certain epileptic phenomena, but also indicate that neurons in the CNS are subjected to the tonic regulation of endogenous adenosine to maintain homeostasis.

Finally, the possibility that focal activation of adenosine receptors in the PPC could protect against seizures induced by systemically administered

bicuculline was investigated. The intravenous, time-infusion technique was employed for measuring the dose of bicuculline required to elicit a minimal seizure in rat. Bilateral pretreatments of the adenosine analog NECA elevated the bicuculline seizure threshold, suggesting that the prepiriform cortex could be an action site of adenosine to inhibit seizures initiated with intravenous bicuculline. This observation provides additional support for the hypothesis that the A_1 adenosine receptor population in the prepiriform cortex plays a fundamentally important role in modulation of seizure susceptibility in the CNS.

In conclusion, the prepiriform cortex appears to be an important anatomic locus for mediating the anticonvulsant effect of adenosine in the CNS. The sensitivity of KA and BMI-induced seizures in the PPC to suppression by activation of adenosine receptors, described herein, suggests that both pre- and postsynaptic mechanisms underlie the anticonvulsant action of adenosine. Pharmacological characterization indicates that the A_1 adenosine receptor subtype is associated with the regulation of the neuronal circuitry in the PPC. The results obtained by manipulation of endogenous adenosine levels strongly support the hypothesis that adenosine is an endogenous antiepileptogenic agent in this paleocortical brain area. The clinical implications from the present investigation, therefore, are that adenosine receptor antagonists such as caffeine should be cautiously used or contradicted in epileptic patients, and that adenosine A_1 agonists and compounds that manipulate endogenous adenosine levels, such as an adenosine kinase inhibitors and the nucleoside blockers, may represent a novel class of antiepileptic drugs.

FIGURE VI-1 A schematic picture of the presumed anticonvulsant mechanisms of adenosine in the rat prepiriform cortex. Neuronal circuitry underlying the development of convulsions from the prepiriform cortex (PPC) modified from Piredda and Gale (1986).

Presynaptic

(+): KA (kainic acid) causes release of excitatory amino acids (Glu and Asp);

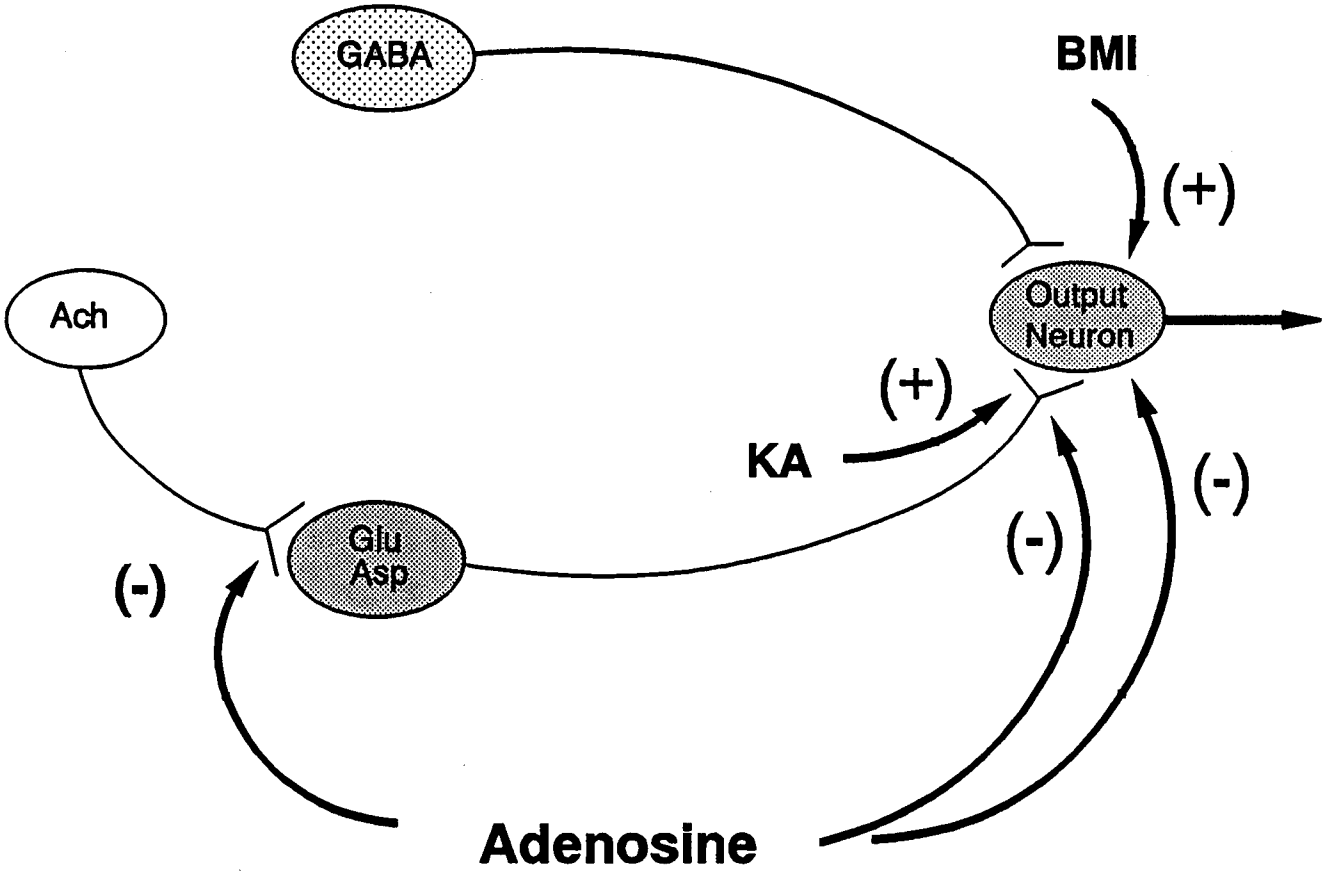
(-): adenosine inhibits release of excitatory amino acids.

Postsynaptic

(+): BMI (bicuculline methiodide) excites output neuron via $GABA_A$ receptor blockade;

(-): adenosine inhibits output neuron via adenosine receptor activation.

FIGURE VI-1



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